DOCTOR OF PHILOSOPHY

# Human mesenchymal stromal cell regulation of pulmonary macrophage populations in the Acute Respiratory Distress Syndrome

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# Human Mesenchymal Stromal Cell Regulation of Pulmonary Macrophage Populations in the Acute Respiratory Distress Syndrome

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## Declaration

I declare that the work presented in this thesis and its composition is based entirely on my own work and that all results and statements presented herein are correct and to the best of my knowledge. None of the material in this thesis has been submitted for which a degree has been or will be conferred by any other university or institution nor has this thesis already been submitted to obtain a degree by this university.

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P.S. I'll try not to forget anyone mentioned here when I'm giving my Nobel Prize acceptance speech while doing a little jig.

## Abstract

The Acute Respiratory Distress Syndrome (ARDS) is a devastating clinical disorder characterised by excessive inflammation in the alveolar compartment resulting in oedema of the airspaces due to loss of integrity in the alveolar epithelial-endothelial barrier. ARDS is associated with high mortality rates and there are currently no effective pharmacological therapies available. Human Mesenchymal Stromal Cells (hMSCs) are a promising candidate therapy which are currently being investigated in clinical trials for ARDS. However their mechanisms of effects in lung injury are not fully elucidated. A fuller understanding of these mechanisms may highlight novel therapeutic targets, identify potency assays to inform hMSC donor selection or biomarkers to assess their efficacy in clinical samples. The alveolar macrophage (AM) is key to orchestrating the inflammatory response in lung injury highlighting the AM as an ideal therapeutic target. hMSCs are known for their immunomodulatory capacity and so it was hypothesised that hMSCs could modulate human macrophage function to adopt a more anti-inflammatory phenotype. The aims of this project were to investigate the effect of hMSCs on human macrophage phenotype and function and to determine the mechanisms of these effects. hMSCs were able to promote an anti-inflammatory (M2-like) macrophage phenotype in lipopolysaccharide or ARDS patient bronchoalveolar lavage fluid-treated human macrophages. This phenotype was characterised by a dampened inflammatory cytokine secretory profile, increased expression of the classical M2 macrophage marker CD206 and enhanced phagocytic capacity. Blocking hMSC-derived extracellular vesicle (EV) uptake by human macrophages using anti-CD44 antibody reversed these effects. Moreover, the adoptive transfer of murine AMs which had been pre-treated with hMSC-derived EVs was protective in endotoxin-induced lung injury *in vivo* highlighting the AM as a key cellular mediator of hMSC beneficial effects. A proportion of hMSC-EVs were found to contain mitochondria which were transferred to human macrophages *in vitro* facilitating hMSCs modulatory effects through the enhancement of macrophage mitochondrial oxidative phosphorylation. These data report a novel mechanism by which hMSCs modulate macrophage phenotype in *in vitro* and *in vivo* models of ARDS.

## Presentations and publications

### Original article publications

Megan Jackson, *Thomas Morrison*, Declan Doherty, Daniel McAuley, Michael Matthay, Adrien Kissenpfennig, Cecilia O'Kane and Anna Krasnodembskaya. Mitochondrial Transfer via Tunneling Nanotubes (TNT) is an Important Mechanism by which Mesenchymal Stem Cells Enhance Macrophage Phagocytosis in the *in vitro* and i*n vivo* Models of ARDS. Stem Cells, 29 April 2016, 34 (8): 2210-2223.

### Review publications

*Thomas Morrison,* Daniel McAuley, Anna Krasnodembskaya. Mesenchymal Stromal Cells for Treatment of the Acute Respiratory Distress Syndrome: The Beginning of the Story. Journal of the Intensive Care Society, 21 May 2015, 16(4): 320-329.

### Published abstracts

*Thomas Morrison,* Megan Jackson, Adrien Kissenpfennig, Cecilia O'Kane, Daniel McAuley and Anna Krasnodembskaya. S63 - Human Mesenchymal Stromal Cell regulation of human macrophages in *in vitro* models of the Acute Respiratory Distress Syndrome. Thorax, 2015, 70: Suppl 3 A38.

### Publications in preparation

*Thomas Morrison,* Megan Jackson, Daniel McAuley, Michael Matthay, Adrien Kissenpfennig, Cecilia O'Kane and Anna Krasnodembskaya. Mitochondrial Transfer through Mesenchymal Stromal Cell-derived Microvesicles Modulates Macrophage Polarisation and Function by Promoting Oxidative Phosphorylation in *in vitro* and *in vivo* models of ARDS. In preparation for submission to the American Journal of Respiratory and Critical Care Medicine.

**Oral/Poster presentations at national/international conferences**

*Title: Human Mesenchymal Stromal Cell Regulation of Macrophage Populations in in vitro Models of the Acute Respiratory Distress Syndrome.*

- Oral presentation, British Thoracic Society, London UK, December 2015.
- Poster presentation, 3rd International ARDS Conference, Belfast UK, August 2015.
- Poster presentation, Stem Cells, Cell Therapies and Bioengineering in Lung Biology and Lung Disease Conference, Vermont USA, July 2015.
- Poster presentation, 3rd Annual REMERGE Symposium, Belfast UK, June 2015.
- Poster presentation, 2nd Annual REMERGE symposium, Belfast UK, July 2014.
- Poster presentation, Irish Society for Immunology, Dublin Ireland, September 2014.
- Poster presentation, International Congress of Immunology, Milan Italy, August 2013.

## Abbreviations

| | |
|---|---|
| ADP | Adenosine diphosphate |
| AFC | Alveolar fluid clearance |
| AM | Alveolar macrophage |
| Ang-1 | Angiopoietin-1 |
| AP-1 | Activator protein-1 |
| ARDS | Acute respiratory distress syndrome |
| ATG5 | Autophagy-related gene 5 |
| ATP | Adenosine triphosphate |
| BAL | Bronchoalveolar lavage |
| BALF | Bronchoalveolar lavage fluid |
| BCA | Bicinchoninic acid assay |
| BMDMC | Bone marrow-derived mononuclear cell |
| cAMP | Cyclic adenosine monophosphate |
| CD40, 80 etc. | Cluster of differentiation |
| cIAP | Cellular inhibitor of apoptosis |
| CM | Culture medium |
| COPD | Chronic obstructive pulmonary disease |
| COX1,2 | Cyclooxygenase-1, 2 |
| CREB | cAMP response element-binding protein |
| DAMP | Danger-associated molecular patterns |
| DGCR8 | DiGeorge critical region 8 |
| ECM | Extracellular matrix |
| *E. coli* | *Escherichia coli* |
| ELISA | Enzyme-linked immunosorbent assay |
| ECSIT | Evolutionarily conserved signalling intermediate in Toll pathways |
| ENaC | Epithelial sodium channel |
| ESC | Embryonic stem cells |

| | |
|---|---|
| EtBr | Ethidium bromide |
| EVs | Extracellular vesicles |
| FCCP | Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone |
| FGF | Fibroblast growth factor |
| FOXO3 | Forkhead box O3 |
| G-CSF | Granulocyte colony-stimulating factor |
| GM-CSF | Granulocyte macrophage colony-stimulating factor |
| GVHD | Graft versus host disease |
| HA | Hyaluronic acid |
| HIF | Hypoxia-inducible factor |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antigen |
| HSCT | Haematopoietic stem cell transplant |
| ICAM-1 | Intercellular adhesion molecule-1 |
| ICU | Intensive care unit |
| IDO | Indoleamine 2,3-dioxygenase |
| IL-6, 8, 10 etc. | Interleukin |
| IL-1ra | IL-1 receptor antagonist |
| IFNγ | Interferon gamma |
| IM | Interstitial macrophages |
| IN | Intranasal/intranasally |
| iNOS | Inducible nitric oxide synthase |
| IP | Intraperitoneal/intraperitoneally |
| IPF | Idiopathic pulmonary fibrosis |
| IRAK | Interleukin-1 receptor-associated kinase 1 |
| IRF | Interferon regulatory factor |
| Jmjd3 | Jumonji domain containing-3 |
| KGF | Keratinocyte growth factor |
| KLF4 | Krüppel-like factor 4 |

| | |
|---|---|
| *K. pneumoniae* | *Klebsiella pneumoniae* |
| LAMP1 | Lysosome-associated membrane protein 1 |
| LCoR | ligand-dependent nuclear receptor corepressor |
| LDH | Lactate dehydrogenase |
| LFA-1 | Leukocyte function-associated antigen-1 |
| LIF | Leukaemia inhibitory factor |
| LL-37 | Human cathelicidin protein |
| LPS | Lipopolysaccharide |
| MCP | Monocyte chemoattractant protein |
| MDA-5 | Melanoma differentiation-associated gene 5 |
| MDM | Monocyte-derived macrophage |
| MFI | Median fluorescence intensity |
| MHC | Major histocompatibility complex |
| MIP | Macrophage inflammatory protein |
| MMP | Matrix metalloproteinase |
| MSC | Mesenchymal stromal cell |
| *M. tuberculosis* | *Mycobacterium tuberculosis* |
| MVs | Microvesicles |
| MyD88 | Myeloid differentiation factor 88 |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NETs | Neutrophil extracellular traps |
| NF-κB | Nuclear factor-kappa B |
| NIBTS | Northern Ireland Blood Transfusion Service |
| NIH | National Institute of Health |
| NK cell | Natural killer cell |
| N-PCP-III | N-terminal peptide for type III procollagen |
| NRF | Nuclear respiratory factors |
| OCR | Oxygen consumption rate |
| *P. aeruginosa* | *Pseudomonas aeruginosa* |

| | |
|---|---|
| PAF | Platelet-activating factor |
| PAMP | Pathogen-associated molecular pattern |
| *P. carinii* | *Pneumocystis carinii* |
| PEEP | Positive end-expiratory pressure |
| PGC-1β | PPARγ-coactivator-1β |
| PGE2 | Prostaglandin E2 |
| PI3K | Phosphoinositide-3-kinase |
| Poly(I:C) | polyriboinosinic:polyribocytidylic acid |
| PPARγ | Peroxisome proliferator-activated receptor-γ |
| PS | Penicillin-streptomycin |
| Rac1 | Ras-related C3 botulinum toxin substrate 1 |
| RANTES | Regulated on activation, normal T cell expressed and secreted |
| RBPJ | Recombination signal binding protein for immunoglobulin kappa J region |
| RIG-1 | Retinoic acid-inducible gene-1 |
| Rip1 | Receptor interacting protein 1 |
| RISC | RNA-induced silencing complex |
| ROS | Reactive oxygen species |
| RPMI | Roswell Park Memorial Institute |
| RT-PCR | Real time polymerase chain reaction |
| *S. aureus* | *Staphylococcus aureus* |
| SD | Standard deviation |
| SDF-1 | Stromal cell-derived factor-1 |
| SLE | Systemic lupus erythematosus |
| SOCS | Suppressor of cytokine signalling |
| Sox9 | SRY-related high mobility group-Box gene 9 |
| *S. pneumoniae* | *Streptococcus pneumoniae* |
| STAT | Signal transducer and activator of transcription |
| sTNFR | Soluble tumour necrosis factor receptor |

| | |
|---|---|
| *S. typhimurium* | *Salmonella typhimurium* |
| TAM | Tumour-associated macrophage |
| TFEB | Transcription factor EB |
| TGFβ | Transforming growth factor beta |
| TLR | Toll-like receptor |
| TNFα | Tumour necrosis factor alpha |
| TNFAIP3 | Tumour necrosis factor alpha-induced protein 3 |
| TNT | Tunneling nanotubule |
| TRAF6 | TNF receptor-associated factor 6 |
| TSG6 | TNF stimulated gene protein-6 |
| V-CAM | Vascular cell adhesion molecule |
| VE-cadherin | Vascular endothelial-cadherin |
| VEGF | Vascular endothelial growth factor |

# Chapter 1

# Introduction

## 1.1 Acute Respiratory Distress Syndrome (ARDS)

### 1.1.1 ARDS definition and epidemiology

ARDS is a clinical disorder with a range of aetiologies that incites a powerful inflammatory response. Extensive damage to the alveolar spaces in the distal lung leads to the development of hypoxia and pulmonary oedema[1]. Originally described by Ashbaugh *et al* in 1967 as the "acute onset of tachypnoea, hypoxemia, and loss of compliance"[2], ARDS has been the subject of extensive research by basic scientists and clinicians alike. Currently, diagnosis requires that symptoms develop within seven days of the insult, bilateral lung infiltrates must be evident through chest imaging and that hydrostatic oedema and cardiac failure are not solely responsible[3]. Mortality rates vary from 25-40% and are dependent on the severity of the condition[3-6]. These statistics have improved with time; a reflection on improvements in supportive care and particularly the mitigation of ventilator-induced lung injury (VILI) through the use of low-volume mechanical ventilation[4, 7, 8]. An effective pharmacological treatment has not yet been identified[9]. Translation of efficacy in preclinical models to benefit in patients with ARDS has proven difficult[10-14]. Stratified medicine aimed at identifying risk factors to inform tailored therapy for certain patient groups is developing interest[15]. For example, prone positioning in severe ARDS reduced mortality at 28 and 90 days[16]. Prone positioning also seems to benefit patients with severe hypoxemia[17]. A heterogeneous patient population and complex pathophysiology makes identification of novel therapies for ARDS very difficult. There is greater promise in putative therapies which target many elements of ARDS pathophysiology.

### 1.1.2 ARDS pathophysiology

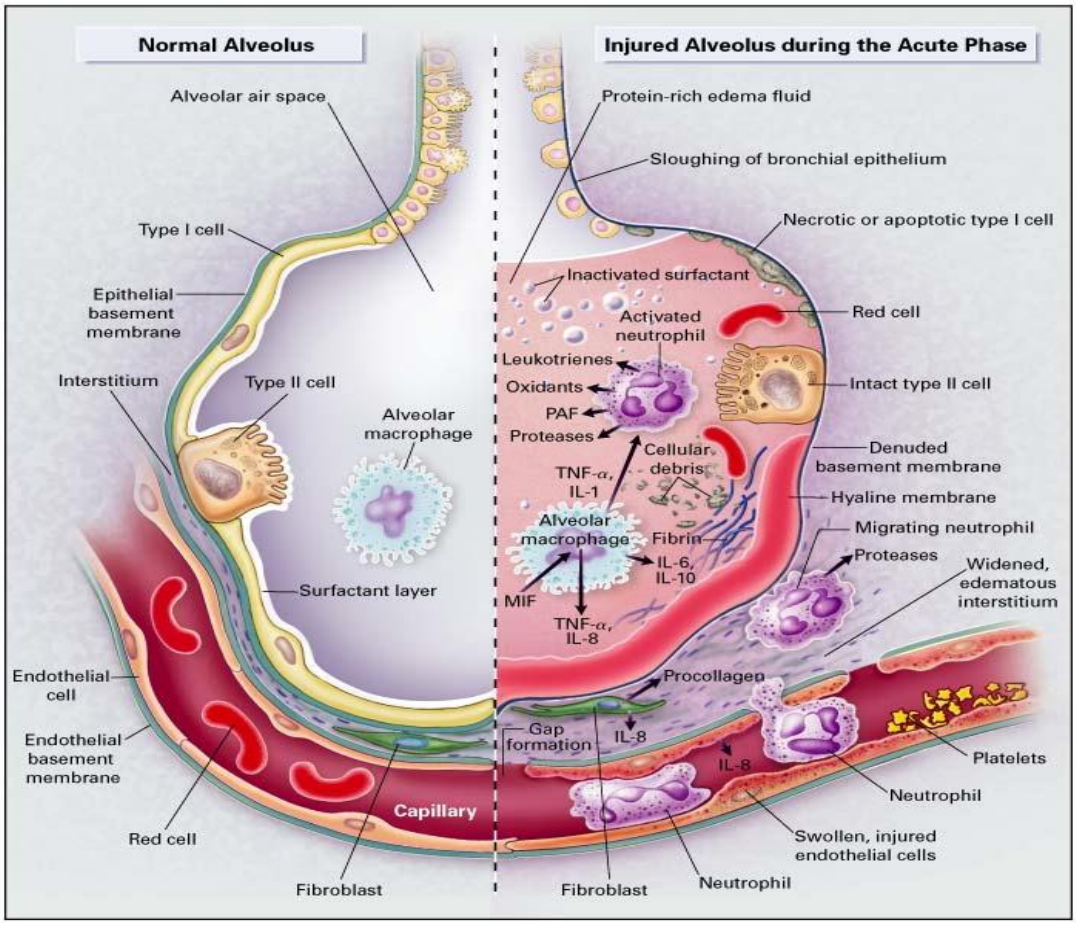
ARDS affects the bronchoalveolar compartment of the lung. The alveoli themselves are made up of epithelial cells and are surrounded by the endothelia of the pulmonary microvasculature. The alveolar macrophage (AM) resides in the airspaces and is the resident immune cell of the lung. After insult, such as infection or trauma, an inflammatory cascade begins with the AM. Nuclear Factor-kappa B (NF-κB), a key inflammatory transcription factor, is activated in the AMs of patients with ARDS with

subsequent secretion of the pro-inflammatory cytokines interleukin-6 (IL-6) and IL-8[18, 19]. The inflammatory cascade, and in particular IL-8 production, recruits neutrophils from the peripheral blood which propagate the inflammation and cause further damage. Notably the survival of neutrophils in ARDS patients is prolonged by alveolar granulocyte and granulocyte/macrophage colony-stimulating factors (G-CSF and GM-CSF) which further facilitates these harmful effects[20].

These events result in the death of alveolar and distal epithelial cells, which is mediated by neutrophil-derived soluble Fas ligand[21, 22]. Neutrophils also produce Neutrophil Extracellular Traps (NETs) which constitutes an important antimicrobial function but are also associated with epithelial and endothelial cell cytotoxicity[23]. Pulmonary microvasculature permeability is strongly associated with the robustness of endothelial adherens junctions. A major component of these junctions is Vascular Endothelial-cadherin (VE-cadherin) whose phosphorylation dictates the extent of permeability and leukocyte extravasation[24]. VE-cadherin is destabilised by Tumour Necrosis Factor alpha (TNFα), another pro-inflammatory cytokine produced by macrophages[25-27]. The resultant loss of integrity of the alveolar barrier allows a protein-rich exudate from the blood to enter the airspaces thereby hindering gas exchange[1]. Alveolar Fluid Clearance (AFC), the active process of pumping fluid out of the airspaces, is impeded in patients of ARDS[28]. The hallmark features of the alveolar compartment during ARDS are depicted in **Figure 1.1.2**. Following lung injury, there are two primary outcomes; regeneration or fibrosis. In regeneration, remaining type II pneumocytes of the alveolar epithelium proliferate and differentiate into type I pneumocytes restoring the epithelial barrier[29]. Whilst it is believed that these type II cells are primarily responsible for repopulating injured alveolar epithelium, there is evidence of progenitor cells found in more proximal regions of the lung which could also contribute[30]. Should the injury persist and regeneration be hindered then fibrosis will occur. Fibrosis involves excessive collagen deposition by mesenchymal cells forming fibrous tissue in place of the injured alveolus[31, 32]. The fibrotic response has been demonstrated in the early stages of ARDS with the detection of elevated levels of N-terminal peptide for type III procollagen (N-PCP-III) in patient bronchoalveolar lavage fluid (BALF) and serum compared to control patients. This study also observes higher N-PCP-III levels in non-survivors compared to survivors

of ARDS[33]. The mechanisms determining whether or not a patient follows the path of regeneration and recovery are not fully understood but there are likely many elements involved including risk factors such as age, gender, ethnicity and disease severity[34-36].

*Figure 1.1.2: The alveolar compartment in health vs. ARDS*




*Ware, L.B. and Matthay, M.A., 2000[1]*

## 1.2 Mesenchymal Stromal Cells (MSCs)

### 1.2.1 Introduction

MSCs are a heterogeneous cell population found in many adult tissues including bone marrow, skin, adipose tissue, placenta and skeletal muscle[37-39]. MSCs may differentiate into a variety of specialised cell phenotypes and have great potential in regenerative medicine applications[40-42]. Currently MSCs are characterised using the

criteria proposed by the International Society for Cellular Therapy in 2006. This position statement dictates that MSCs must be plastic adherent, express mesenchymal markers including CD73, CD90 and CD105 but not haematopoietic markers such as CD14, CD34 and CD45 and finally that they have the capacity to differentiate into osteoblasts, chondroblasts and adipocytes *in vitro*[43]. MSCs possess a number of qualities which are greatly advantageous for therapeutic application. Donor tissue and organ mismatching and subsequent rejection is a major limitation in transplantation medicine. Some evidence would suggest that MSCs have limited immunogenicity and that allogeneic administration is well tolerated[44, 45].

In homeostasis MSCs lack major histocompatibility complex (MHC) class II expression but this is induced by interferon gamma (IFNγ) treatment. MSCs do not express cluster of differentiation (CD) 40, 80 or 86, each of which are co-stimulatory molecules involved in T cell activation, contributing to recipient tolerance to allogeneic donation of MSCs[46]. The extent of immunogenicity of MSCs is a topic of controversy; there are reports that MSCs elicit immune responses in non-matched hosts[47, 48]. These inconsistencies could be explained by differences in the source of MSCs or the conditions under investigation. It is apparent then that MSCs are not tolerated in all cases highlighting the need for more study. An additional concern for stem cell therapies is the potential for the development of tumours as has been reported with embryonic stem cells[49]. MSCs taken from adipose tissue and cultured *in vitro* have been reported to exhibit genetic stability for at least 12 passages with no evidence of tumourigenicity when given to immunodeficient mice intravenously (IV)[50, 51]. This was corroborated by Bernardo *et al* demonstrating human bone marrow-derived MSCs cultured until passage 25 with no change in telomerase activity and subsequently telomere length[52]. Conversely, another group reported malignant transformation after long term culture (up to 105 weeks) of human MSCs (hMSCs)[53]. Moreover, MSCs were shown to promote metastasis of breast cancer cells[54]. The tumorigenic potential of MSCs is another area of debate; again this will require more in depth investigation and highlights the necessity for long term studies of MSC administration *in vivo* as well as long-term follow up of patients that may receive MSC therapy.

MSCs have potent immunomodulatory potential; they may influence T lymphocytes, dendritic cells, natural killer (NK) cells and others conferring them with regulatory functions in both innate and adaptive immunity[55, 56]. MSCs inhibit T cell proliferation and induce regulatory phenotypes in T cells through promotion of anti-inflammatory macrophages[57, 58]. They dampen NK cell responses towards HLA class I-expressing cells and inhibit monocyte differentiation into dendritic cells[59, 60]. Through these actions MSCs may prolong allogeneic graft survival given in different settings[61, 62]. Cruz *et al* show that MSCs, monocytes and macrophages dampen hyperresponsiveness in Th2/Th17-mediated allergic airway inflammation modelling asthma[63]. MSCs facilitated this effect through the induction of regulatory T cells and hindering Th17 function[64]. MSCs have the inhernetn ability to home to sites of injury after systemic administration IV. Stromal cell-derived factor-1 (SDF-1) is released by injured cells[65, 66]. SDF-1 is a ligand for the chemotactic receptor CXCR4 which is expressed on a small proportion of MSCs and mediates their homing to these sites[67]. Furthermore, SDF-1 binding enhances Akt kinase signalling thereby augmenting paracrine factor production highlighting the ability of MSCs to respond to signals in their environment[68]. Extravasation of MSCs begins with adhesion and rolling along blood vessels and is mediated by vascular cell adhesion molecule-1 (VCAM-1) and P-selectin found on endothelial cells. MSCs will form clusters with neutrophils and platelets which effectively slows MSCs down in the bloodstream to assist in adhesion and rolling[69, 70]. MSCs appear to exit the vasculature by unconventional means. In a zebrafish model, Allen *et al* demonstrate the extravasation of MSCs by a process that they termed 'angiopellosis' characterised by single MSCs or clusters being enveloped by endothelial cell cytoplasmic projections and actively extruded into the peripheral tissues. Angiopellosis differs from conventional white blood cell diapedesis because the endothelial cells undertake the morphological changes required for the transition while the MSC remains relatively unchanged; this is a stark contrast to diapedesis where the white blood cell would contort itself allowing it to squeeze through the endothelial cell junctions[71]. MSCs are relatively large cells and are known to become lodged in the pulmonary microvasculature. Gao *et al* reported MSC sequestration to the lungs after intravenous infusion with smaller amounts localising to the spleen and liver. Eventually MSCs were cleared from the lung and more were found preferentially in the liver[72].

### 1.2.2 MSCs in preclinical models of ARDS

MSCs are being tested in a number of inflammatory conditions including myocardial infarction, acute renal failure and sepsis with evidence of therapeutic efficacy[73-75]. This is also true of ARDS and lung injury where MSCs are also under thorough investigation. Intrapulmonary (IP) treatment of mice with endotoxin-induced lung injury using murine MSCs reduced alveolar epithelial barrier permeability and oedema improving survival[76]. MSCs given intratracheally (IT) four hours after injury, were protective in an *Escherichia coli* (*E. coli)* pneumonia model of lung injury[77]. Umbilical cord-derived MSCs given IV 24 hours after injury reduced inflammation and fibrosis in a bleomycin-induced lung injury model[78]. Both human and rat MSCs improve repair of lung tissue after ventilator-induced lung injury[79, 80]. In a sheep model of ARDS triggered by smoke inhalation and bacterial pneumonia human MSCs reduced pulmonary oedema and improved oxygenation[81]. Lee *et al* demonstrate hMSCs ability to enhance alveolar fluid clearance in a human *ex vivo* lung perfusion model of endotoxin and bacteria-induced injury. [82, 83]. MSCs show promise in sepsis models enhancing survival through promotion of bacterial clearance via augmented monocyte phagocytosis and induction of IL-10 production by macrophages[73, 84, 85]. Such studies are of particular importance given that sepsis is a leading cause of ARDS with one of the highest mortality rates[86].

### 1.2.3 MSC mechanisms in lung injury

One of the biggest advantages of cellular therapies including MSCs is their ability to adapt and respond to cues in their environment. MSCs may exert a therapeutic effect targeting many elements of ARDS. MSCs are already known to act through numerous mechanisms. Because the alveolar epithelial barrier loses integrity during lung injury it was proposed that MSCs, having extensive differentiation potential could engraft into the alveolar epithelium. Such engraftment into the lung epithelium does occur, although it is an uncommon event with reports of less than 5% engraftment and so it is unlikely that this contributes significantly to their effects in these preclinical models[76, 87-91]. *Most evidence points towards the importance of paracrine factor secretion in exerting these effects (which forms the rationale for investigating paracrine mechanisms in this study).* In a number of reports MSC secretion of

keratinocyte growth factor (KGF) mediated the restoration of alveolar fluid clearance through the rescue of epithelial sodium channel (ENaC) activity[82, 92, 93]. Angiopoietin-1 (Ang-1), also produced by MSCs, is also known to play a role in reducing alveolar epithelial permeability[94].

Researchers had concerns that the anti-inflammatory effects of MSCs could cause complications in treatment of ARDS patients by hindering the host's ability to fight infection in sepsis or pneumonia-induced ARDS. Importantly, models of sepsis and bacterial pneumonia consistently report reduced inflammation and bacterial loads in MSC-treated animals. MSC immunomodulation also affords innate immune cells with improved antimicrobial capacity. Two different septic models show that MSCs increase the phagocytic capacity of blood monocytes and CD11b positive cells[73, 84]. MSC enhancement of phagocytosis also extends to neutrophils; Hall *et al* report increased neutrophil activity in a cecal ligation and puncture model. In this study, depletion of neutrophils prevented MSC beneficial effects[95]. In *ex vivo*-perfused human lungs injured with *E. coli*, KGF production by MSCs enhanced AM phagocytosis, reducing bacterial load which was associated with higher granulocyte-macrophage colony stimulating factor (GM-CSF) levels in the BALF[83]. MSCs produce antimicrobial proteins such as the human cathelicidin, LL-37 and lipocalin-2 which blocks iron uptake by bacteria[77, 96]. TNF stimulated gene protein-6 (TSG6) is contributes to immunomodulation by MSCs which is implicated in their therapeutic effects in myocardial infarction and wound healing[97, 98]. In endotoxin-induced lung injury MSCs amplify TSG6 production which was necessary for their anti-inflammatory effects[99]. MSCs which are cultured on non-adherent surfaces form spheroid aggregates initiating caspase-dependent IL-1 signalling which augments their TSG6 production as well as other anti-inflammatory mediators[100]. The formation of such clusters may explain the tendency for MSCs to become trapped in the pulmonary microvasculature. MSCs produce a number of anti-inflammatory cytokines; a subset of MSCs produce IL-1 receptor antagonist (IL-1ra) which blocked pro-inflammatory TNFα and IL-1α activity in bleomycin-induced lung injury[101]. Prostaglandin E2 (PGE2) is another mediator of MSC regulatory functions that encourages macrophages to produce anti-inflammatory IL-10 in sepsis modelled by cecal ligation and puncture[85]. Interestingly MSCs may also produce pro-inflammatory mediators

after certain environmental cues. MSCs can secrete both IL-6 and IL-8, which are associated with poor prognosis in ARDS[102, 103]. IL-6 is a promiscuous cytokine with pro-inflammatory and anti-inflammatory functions[104-106]. IL-6 contributes to the beneficial effects of MSCs in endotoxin-induced lung injury[107]. The role of MSC-derived IL-8 in lung injury is unknown, although IL-8 was shown to upregulate vascular endothelial growth factor (VEGF) production by MSCs proposing a potential pro-angiogenic element to the MSC effect[108]. VEGF is well known for its pro-survival effects on vascular endothelial cells[109, 110]. Interestingly, TSG6 may bind IL-8 preventing it's activity[111]. TSG6 may play a role in hindering neutrophil recruitment in this manner.

MSCs also produce EVs, membrane-bound compartments which contain biologically active molecules[112, 113]. These EVs are responsible for many of the modulatory effects of MSCs and will be discussed in detail in **Section 1.5.3**. A number of groups have reported mitochondrial transfer from MSCs to other cell types using tunnelling nanotubules (TNTs). One group was able to observe mitochondrial intercellular trafficking from MSCs to epithelial cells with the use of TNTs which was regulated by the Rho-GTPase Miro1. Importantly, the overexpression of Miro1 resulted in enhanced mitochondrial transfer and was associated with enhanced therapeutic effect of MSCs in allergen-induced asthma models[114]. Liu *et al* then demonstrated the formation of TNTs between MSCs and endothelial cells; the TNTs also facilitated mitochondrial transfer which enhanced survival of the endothelial cells in an ischaemia-reperfusion injury model *in vitro*[115]. Our group has recently published a paper which demonstrated the ability of MSCs to transfer their mitochondria to human macrophages via these TNTs. We demonstrated that the transfer of mitochondria was responsible for their enhancement of phagocytosis and clearance of bacteria in human macrophages *in vitro* and reduced bacterial counts in the lung in an *in vivo E. coli*-induced lung injury model[116]. Studying the bioenergetics of human macrophages in MSC co-culture showed that MSCs were able to increase mitochondrial basal respiration and ATP turnover in these macrophages, which may offer an explanation for their increased phagocytic capacity. When TNT formation is prevented using cytochalasin B the improvement in bacterial clearance *in vivo* is lost, but interestingly CFU counts in lung homogenate are not only reversed but actually exceed the CFUs

of mice not receiving any MSCs. This suggests that MSCs without the ability to transfer mitochondria, and so not enhancing phagocytosis, actually worsen the host's ability to combat infection. This is likely a result of blocking the antimicrobial functions of MSCs without affecting their immunoregulatory properties, resulting in a more hospitable environment for bacteria. The benefits of TNT formation extends beyond *E. coli*-induced pneumonia models of lung injury. MSCs were shown to generate TNTs allowing transfer of mitochondria to bronchial epithelial cells in cigarette smoke-induced lung injury, modelling chronic obstructive pulmonary disease (COPD)[117]. Interestingly, MSCs are not always the mitochondrial donors but may be the recipients; co-culture of MSCs with vascular smooth muscle cells resulted in enhanced proliferation of MSCs and was also facilitated by TNT-mediated mitochondrial transfer[118]. Although the mechanisms of effect of MSCs in the context of lung injury continue to be defined, it is already apparent that their actions are multifaceted, impacting on the numerous components of the pathophysiology of ARDS.

### 1.2.4 MSCs for the treatment of ARDS

MSCs are being investigated in clinical trials for a number of conditions including ARDS[119-128]. Simonson *et al* treated two patients with severe refractory ARDS with $2x10^6$ cells/kg of MSCs on a compassionate use basis. They performed a thorough analysis of the efficacy of these MSCs and performed *in vitro* functional assays to determine associations between their *in vitro* and *in vivo* performances. Both patients showed improvement measured by pulmonary and systemic inflammatory markers, epithelial apoptosis and pulmonary oedema. The same MSCs performed well in *in vitro* potency assays suppressing T cell activation and promoting regulatory T cells and monocytes[129]. While these are the results from only two case studies, they do provide encouraging evidence of the potential efficacy of MSCs in severe ARDS and highlight the merit of potency assays in identifying optimal MSC preparations. A randomised, placebo controlled pilot study testing safety and feasibility of allogeneic adipose-derived MSCs ($1x10^6$ cells/kg of predicted body weight) in ARDS patients (PF ($PaO_2/FiO_2$) ratio < 200mmHg) reported that MSC treatment did not incite any adverse events[130]. While this was a small cohort there were no apparent effects on

length of hospital stay or the number of ventilator or intensive care unit (ICU)-free days. Serum surfactant protein-D (marker for type II pneumocyte injury) levels trended downwards but conclusions in efficacy in this study tentative. A multicentre, open-label, dose-escalation study also tested safety and feasibility of allogeneic bone-marrow derived MSCs in moderate-to-severe ARDS (PF ratio < 200mmHg, positive end-expiratory pressure (PEEP) > 8cm $H_2O$). Three different doses of MSCs were investigated: low ($1x10^6$ cells/kg), intermediate ($5x10^6$ cells/kg) and high dose ($10x10^6$ cells/kg). Across all dosing regimens, no association with adverse events were reported[131]. These studies were performed with very small patient numbers and short-term follow-up but still support further investigation of MSCs as a treatment of ARDS in larger scale phase II trials (clinicaltrials.gov, NCT02097641). Long-term follow-up must be carried out to ascertain whether there are any delayed adverse events associated with MSC treatment. Naturally, these pilot studies used single dose regimens. Other clinical trials have investigated repeated dosing with MSCs (including a weekly dose for four weeks for treatment of Crohn's disease[122] or two weekly doses for four weeks in graft versus host disease (GVHD)[127]) where they were well tolerated and resulted in improvement of clinical outcomes. Repeated MSC doses may be safe and potentially more effective than single doses but further study on safety is required before such studies can be approved.

### 1.2.5 Optimisation of MSC therapeutic effects

Preclinical research is essential in determining optimal culture conditions to maximise the therapeutic effects of MSCs. MSC function is affected by several parameters regarding their expansion and administration. The administration route may have significant impact on their effects in lung injury models; IP delivery was proven more effective than IN during lung injury in the neonate[88]. IP injection of MSCs fared poorly relative to intratracheal (IT) or IV application in VILI[80]. The optimal tissue source of MSCs is also under investigation. MSCs may be found in numerous sites throughout the body and have distinctive qualities associated with each[132-134] Comparison of human adipose, bone marrow and umbilical cord-derived MSCs demonstrated that umbilical cord MSCs have are more proliferative and senesce at a lower rate (as measured by p53 and p21 levels)[133]. Umbilical cord-derived MSCs

may then be best suited for regenerative medicine applications. The conditions that MSCs encounter during *ex vivo* expansion and culture influence their function. Hypoxic pre-conditioning (mimicking a key element of ARDS), resulted in enhanced chemotaxis and viability of MSCs as well as increased production of paracrine factors[135]. Hypoxia also resulted in better preservation of the stem/progenitor subset of MSCs which are believed to be the therapeutically active cells of this heterogeneous population[136]. Interestingly, culturing MSCs in ARDS patient serum enhanced IL-1ra and IL-10 production in endotoxin-induced lung injury mice improving outcomes[137]. Zheng *et al* implemented this pre-conditioning method of adipose tissue-derived MSCs in their phase I pilot study in ARDS patients[130].

Artificial manipulation of MSC biology may hold the potential to enhance their therapeutic efficacy. MSC overexpression of soluble IL-1 receptor-like-1 (a decoy receptor for IL-33), enhanced their pro-reparative and anti-inflammatory effects in endotoxin-induced lung injury[138]. IL-33 is constitutively expressed in endothelial and epithelial cells of the lung which is released after injury and triggers inflammation[139, 140]. Increasing Ang-1 production by MSCs using a vector amplifies their anti-inflammatory functions and rescues alveolar barrier permeability to a larger degree in an LPS lung injury model[141]. Even MSC lung engraftment may be augmented by inhibiting the Wnt/β-catenin signalling pathway[142]. Treatment with TLR ligands can influence MSC phenotype. Waterman *et al* demonstrate that TLR3 ligation induces an immunosuppressive MSC phenotype whereas TLR4 ligation promotes pro-inflammatory functions[143]. TLR4 stimulation induced increased IL-6 and IL-8 production and TLR3 ligation upregulated IL-4 and IL-1ra levels. Conversely, another report showed TLR3 stimulation inducing the highest extent of IL-6 and IL-8 production[144]. These contrasting studies tested the effects of TLR ligation on MSCs from different sources, further highlighting the influence of the MSC niche on their biology.

### 1.2.6 MSC handling and quality control

Progression of MSC to the clinic is slowed by incomplete understanding of MSC biology, poor characterisation of the MSC population and the inconsistency of their therapeutic actions. The European Medicines Agency and British Standards Institution both emphasise the requirement for improved characterisation of MSCs, better quality isolation and purification and more complete understanding of their mechanisms of effect. MSC sourcing and culture conditions heavily influence their immunomodulatory capacity; expanding MSCs in the presence of foetal calf serum (FCS) or platelet lysate modulates their ability to inhibit T-cell proliferation[145]. Other factors including the MSC donor's age and serum or glucose content in the culture medium will have profound effects[146-148]. There is a significant effort to optimise MSC isolation and culture techniques[145, 149-152]. Mimicking the extracellular matrix (ECM) of the bone marrow from which MSCs may be derived can promote stem cell functions and proliferation[153]. Carrancio *et al* reported that supplementing culture medium with platelet lysate and exposure to hypoxia resulted increased MSC proliferation rates[154]. Inconsistency in the efficacy of different MSC preparations has highlighted the need for potency assays to allow selection of the best cell product. TSG6 production was considered as a potential potency assay. TSG6 levels correlated positively with MSC anti-inflammatory effects in a model of chemically-induced corneal injury but it negatively correlated with osteogenic differentiation potential[155]. A number of *in vitro* assays used in conjunction were predictive of MSC ability to enhance wound repair[156]. These assays measured cell counts, bromodeoxyuridine incorporation and measurement of ATP levels to assess MSC growth and viability. MSCs scoring highly in these measurements were more effective in enhancing wound repair. Another important question which must be addressed is whether these MSCs may be stored for a period of time before administration or if they must be taken directly from continuous culture. Understandably, there is the concern that freezing and then thawing a preparation of MSCs may affect their efficacy. Indeed, there is some evidence that cryopreservation of MSCs impairs there immunosuppressive properties. Freshly thawed MSCs were shown *in vitro* to be less responsive to IFNγ, regarding upregulation of immunoregulatory indoleamine-2,3-dioxygenase, and less potent in their suppression of T cell proliferation[157]. Conversely, Cruz *et al* showed *in vivo* that both thawed and unthawed MSCs were able to mitigate *Aspergillus* hyphal

extract-induced allergic airway inflammation to a similar degree[158]. It is clear then that further investigation into the effect of cryopreservation on MSC function is required to determine whether or not it is a viable option for the treatment of different conditions. In summation it is critical that the protocols for selection, isolation, purification and expansion of MSCs be optimised for the condition under investigation.

### 1.2.7 Future directions in MSC research

As described above, there are a number of practical hurdles which must be overcome before MSCs may be effectively applied in a clinical setting. Apart from these issues, our understanding of MSC biology is still incomplete. A perspective article produced by Bianco *et al* addresses these ambiguities regarding the origin, identity and functions of MSCs[159]. They argue that the widely used criteria to identify MSCs are too vague and do not necessarily imply stem cell functionality. The authors suggest that the criteria currently used to define MSCs are generally shared by most connective tissue cells and that the *in vitro* differentiation of MSCs under such artificial conditions does not demonstrate stem cell status, rather proper *in vivo* testing is required. Of course MSCs have proven successful in preclinical disease models and are being investigated widely in the clinic. A search on clinicaltrials.gov for 'mesenchymal stromal cells' returned 125 results of studies involving MSCs, many of which are actively recruiting (as of May 2016). While significant progress has been made, we do not yet fully understand the mechanisms of action of MSCs in preclinical models of disease including lung injury. It could be argued that MSCs have progressed too rapidly to clinical trials; there is always an additional risk associated with testing a new therapy in patients which hasn't yet been fully characterised. Prockop *et al* remind us of the success of haematopoietic stem cell transplants (HSCT) and emphasise the arduous and prolonged scientific effort which was required to understand the concept and ultimately allow their effective application in the clinic[160]. For example, one of the important developments in the history of HSCT research was the use of marrow-ablated mouse models[161]. Bone marrow depletion using radiotherapy allowed quantification of the contribution of different haematopoietic stem cell populations and their longevity *in vivo* using different marrow preparations as well as identification

of cell biomarkers[162]. It is studies like these that are lacking in the MSC field; very little is known about the kinetics of MSCs *in vivo* which makes it substantially more difficult to establish an optimal delivery strategy. It is clear that there remains a significant amount of work that needs to be done to develop a complete understanding of MSC function *in vivo.* Whilst early stage clinical trials with MSCs have yielded some promising results in other conditions, there is still no evidence of efficacy of MSCs in patients of ARDS. This will become clearer with larger scale studies but it is likely that the current protocol for the isolation, culture and expansion, dosing and administration of MSCs is not optimal. Indeed, even after HSCT had become standard practice in medicine, its use was still subject to modification and improvement whether it was through superior tissue typing techniques or changes in preconditioning regimens before transplant[163, 164]. Therefore if MSCs do have efficacy in treatment of ARDS, further preclinical research will still be essential for the optimisation of their application in the clinic.

Mechanistic studies in lung injury will not only expand our knowledge of MSC biology but may also allow identification of potency assays or novel therapeutic targets. While there is generally consensus on the criteria for defining MSCs (although those criteria may be subject to change), effective biomarkers of potency are elusive. Osiris Therapeutics Incorporated developed Prochymal®, an industrial scale MSC-like product for a clinical trial in acute GVHD. For selection of MSC batches in this trial they employed soluble TNF receptor-1 quantification and extent of inhibition of IL2Rα expression in T lymphocytes as potency readouts[165]. In this study the therapy did show a high proportion of responders initially but this effect was transient in many patients, with recurring flares being reported. This study only included 32 patients, meaning that we cannot glean any information about the quality of these potency assays. We do however observe that effectiveness in *in vitro* assays is not necessarily predictive of their efficacy *in vivo*. With regards to discovery of alternative therapeutic strategies, EVs represent a good example of how mechanistic studies can identify novel putative therapeutic agents. EVs (to be discussed in detail in **Section 1.4**) are membrane bound compartments produced by cells capable of transporting proteins, mRNAs, miRNAs and other soluble factors as well as organelles. Like most cells, MSCs produce EVs and they have been shown to recapitulate many of the beneficial

effects of the cells themselves[166-171]. Researchers are now investigating the prospect of using MSC EVs in place of MSCs, with the rationale that this anuclear cell-derived product cannot itself form tumours. In addition, there is evidence to suggest that MSC EVs may even hinder tumour progression[166, 172, 173]. While MSCs are not considered to be very immunogenic, EVs do not run as large a risk of eliciting an immune response in the host. Cells may also be augmented to alter the contents of their EVs which adds significant scope to prospect of their use in therapy[174]. Although the number of studies are relatively few, a systematic review of the efficacy of MSC EVs in various disease models lends support to their investigation in patients. Progression towards this end will be accelerated with the contribution of further studies with proper animal randomisation and allocation methods[175].

## 1.3 Macrophages

### 1.3.1 Introduction

The macrophage is a key component of innate immunity and is part of the first line of defence against invading microorganisms. Macrophages are tissue-resident cells derived from peripheral blood circulating monocytes which themselves originate in the bone marrow from myeloid progenitor cells[176]. More recent evidence highlights the presence of embryonic macrophage precursors in many tissues which are capable of self-renewal in the resting state [177, 178]. Macrophages are well known for their phagocytic functions and generation of pro-inflammatory cytokines which is crucial in clearance of pathogens. They also play roles in tissue homeostasis and remodelling, antigen presentation and in resolution of the inflammatory response[179-182]. Whilst canonically associated with cells of the adaptive immune system, it seems that macrophages do possess an element of 'memory' although it may lack the specificity of lymphocyte responses. Lipopolysaccharide (LPS) was shown to phosphorylate the stress-response transcription factor ATF7, releasing it from the chromatin thereby creating a more accessible chromatin structure. LPS-treated cells exhibited increased expression of pro-inflammatory genes and these effects were maintained for three weeks. The 'memory' induced by the priming of macrophages with LPS three weeks earlier was associated with improved resistance to bacterial infection[183].

The macrophage population of the lung is comprised primarily of AMs and interstitial macrophages (IMs) which reside in the connective tissues encompassing the airways. AMs represent approximately 63% of the total pulmonary macrophage population and IMs make up the remainder[184]. Morphological studies show that the AM is larger with a higher nuclear to cytoplasmic ratio compared to IMs which more closely resemble blood monocytes, suggesting that the IM represents an intermediate stage for the differentiation of monocytes into mature AMs[185, 186]. Functionally however, AMs had higher phagocytic capacity than IMs and IMs had more pronounced expression of MHC class Ia and CD54 both of which are integral to antigen presentation[185]. Moreover AMs exhibit a more activated phenotype, with increased production of TNFα and nitric oxide, increased tumoricidal activity and can more efficiently phagocytose opsonised erythrocytes. IMs are more adept in mediating specific immune responses, evidenced by higher expression of MHC class II and production of IL-1 and IL-6[187, 188]. The distinctive functional disparities between the two cell types would argue that they are in fact unique and that IMs are not simply intermediaries between monocytes and AMs. Further complexity is added by the presence of subpopulations of both AMs and IMs. Researchers utilising density centrifugation are able to separate AMs into subgroups, and this is also true of IMs. These subgroups also display functional differences including chemotactic sensitivity, production of PGE2 and phagocytic tendencies[189-191].

AMs exhibit an exceptionally long life-span compared to other types of macrophage. In the resting state, it was found that they may last over 8 months with minimal replacement by the periphery[192]. However, there is accumulating evidence that AM progenitor cells inhabit the lung during early development which are derived from foetal monocytes, These then differentiate and persist throughout adulthood and gradually replenish the AM population[177, 193]. During *Streptococcus pneumoniae (S. pneumoniae)* infection, cell death is accelerated dramatically, with 60% of AMs being replaced in just 24 hours[194]. While some of these AMs are replaced by infiltrating monocytes, the AM-progenitor pool contributes substantially to this repopulation. This would suggest that peripheral monocyte replenishment of macrophages is more of a transient phenomenon during inflammation. AMs are the immune sentinels of the distal lung and reside in the airspaces where they perform crucial functions in

homeostasis and defence. GM-CSF mediates AM differentiation and promotes adhesion, phagocytosis and surfactant catabolism by these cells[195]. The AM is presented with a difficult task; it must be able to mount immune responses to infectious agents whilst minimising collateral damage to the delicate alveolar compartment. At the same time it should avoid overt reactions to harmless particulate matter or antigens which they are exposed to continuously. By nature, the AM is inhibitory and this suppressive state is mediated by epithelial cell expression of αvβ6 integrin allowing activation of latent transforming growth factor beta (TGFβ) which may then promote quiescent AMs[196]. Indeed, AMs are known to commonly reside at the alveolar septal junctions in close proximity to interstitial dendritic cells so that they may inhibit their maturation and antigen presentation capacity[197]. The depletion of AMs by clodronate-containing liposomes resulted in an enhanced response to antigen delivered intratracheally[198].

When a genuine need arises AMs will respond by initiating the inflammatory cascade. Inhaled microbes will bind TLRs on the AM cell surface resulting in their activation[199]. They will subsequently produce inflammatory cytokines and mediate resistance to viral and bacterial infection. This includes production of TNFα, IL-8, IL-6 and IL-1α, which orchestrates the recruitment of neutrophils and monocytes to assist in clearance of pulmonary pathogens such as *Pneumocystis carinii (P. carinii)* and *Pseudomonas aeruginosa (P. aeruginosa)* [200-204]. The AM chemokine profile is also comprised of monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and MIP-1β. Their release was shown to be elicited by the interaction of the hyaluronic acid (HA) receptor CD44 on AMs with low molecular weight fragments of HA, which are produced at sites of inflammation[205]. The AM itself also encounters chemotactic signals in the form of regulated on activation, normal T cell expressed and secreted (RANTES) and GM-CSF from alveolar epithelial cells, the strength of which is amplified when the epithelial cells are exposed to IL-1α[206]. It is well established that there is extensive communication between these two cell types in an inflammatory setting. AM derived-TNFα was able to augment the production of pro-inflammatory mediators by epithelial cells including MCP-1 and IL-6[207, 208]. Some of these cell interactions are contact dependent. A seminal article in the field was produced by Westphalen *et al* (Nature Medicine, 2013)

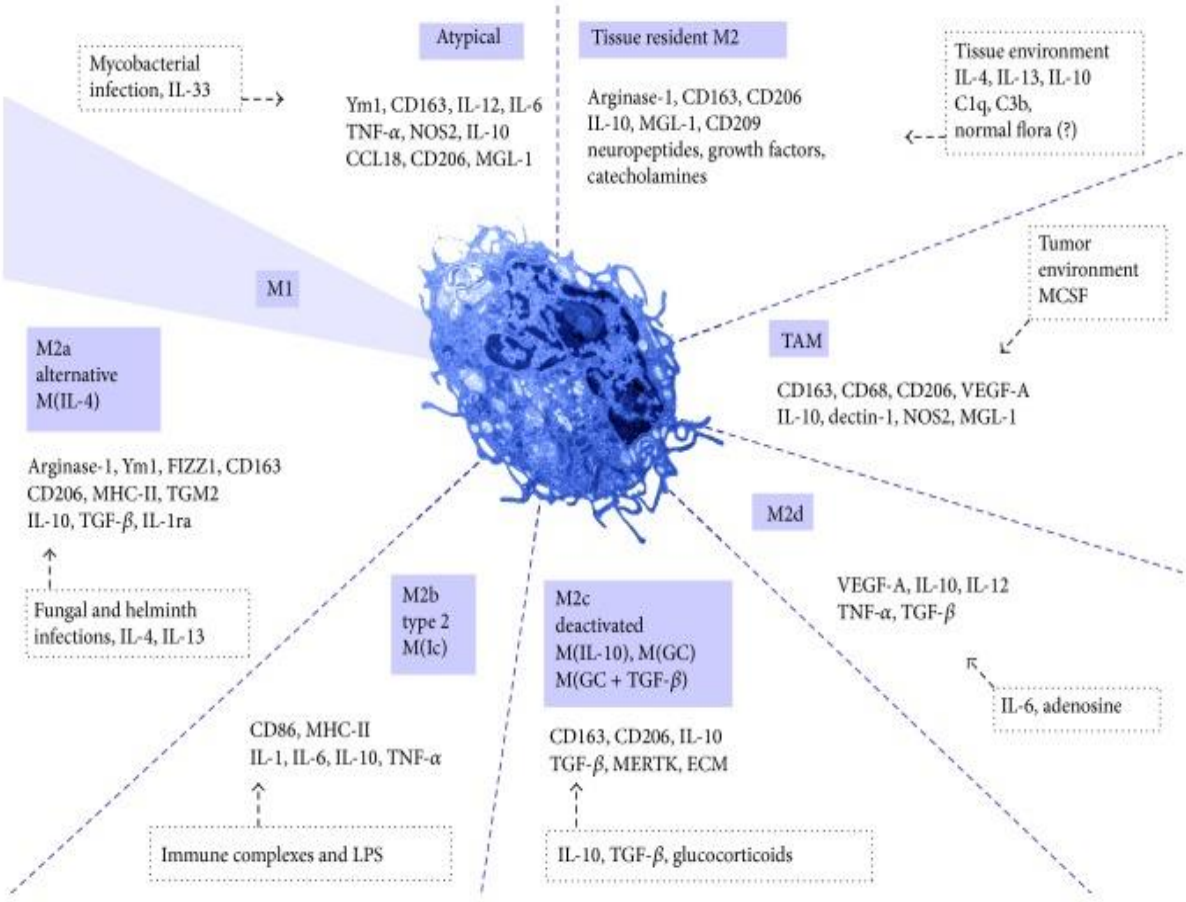
where they demonstrate that many AMs are in fact immobile and remain affixed to the alveolar epithelium. This subset of cells form connexin-43-dependent gap junctions with epithelial cells allowing calcium flow between cells. This interplay and calcium signalling activated the Akt pathway and had an immunosuppressive effect evidenced by the enhanced neutrophil recruitment with an AM-specific connexin-43 knockout[209]. It is clear that cellular communication and co-ordination of responses in the lung are imperative for the effective clearance of pathogens and maintenance of homeostasis for respiratory function.

### 1.3.2 Macrophage polarisation

*Environmental cues and diversity*

The many functions of macrophages are facilitated by the existence of a plethora of different phenotypes. The induction of a given macrophage phenotype is referred to as polarisation. Put simply, macrophages may be driven towards an M1 pro-inflammatory phenotype or an M2 anti-inflammatory phenotype. The M1 class is induced by TLR ligands such as LPS or Th1 cell-derived IFNγ and is chiefly associated with propagation of inflammation and the killing of pathogens. One type of M2 class can be triggered by Th2 cell-derived IL-4 and 13 and is involved in resolution of inflammation[210, 211]. In reality, M1 and M2 macrophages represent the extremes in a broad spectrum of phenotypes, highlighted in **Figure 1.3.2**. These phenotypes are defined by the expression of surface markers, cytokine and chemokine profiles, their phagocytic capacity as well as other functions. The pro-inflammatory M1 macrophage produces cytokines and chemokines including TNFα, IL-12, IL-23, CCL3 and CCL4 and express markers like inducible nitric oxide synthase (iNOS) and CD40[212, 213]. Their pro-inflammatory properties enable them to inhibit tumour cell growth and promote anti-tumour Th1 lymphocyte responses[214]. The M1 macrophage is adept at killing pathogens due to the production of anti-microbial factors and a highly phagocytic phenotype. These macrophages produce reactive nitrogen intermediates and reactive oxygen species through iNOS and nicotinamide adenine dinucleotide phosphate (NADPH) phagocyte oxidase respectively which are crucial to the killing of bacteria and limiting their replication[215-218].

*Fig 1.3.2: The complexity of macrophage polarisation*




*Rőszer, T., 2015*[219]

Many more subtypes of the alternatively activated M2 macrophage phenotype have been described, each induced by different ligands and demonstrating a unique set of functions. Some of the earlier described M2 macrophage subsets were labelled M2a, induced by IL-4 and IL-13, M2b, triggered by co-stimulation with TLR ligands and immune complexes and M2c, generated by IL-10 and glucocorticoids[220]. M2 macrophages are associated with wound healing, tissue remodelling and resolution of inflammation[221-224]. The possible overlap of M1 and M2 macrophage properties is evidenced in the 'regulatory' M2b phenotype of macrophage that is induced by ligation of Fcγ after initial LPS stimulation, which produces less pro-inflammatory IL-12, more anti-inflammatory IL-10 and yet maintains TNFα secretion[225, 226].

*Cell signalling*

The cell signalling pathways and transcriptional regulation involved in regulating macrophage polarisation is complex. Interferon regulatory factor 5 (IRF5) is induced in macrophages by LPS stimulation, and particularly so in those differentiated using GM-CSF. IRF5 induced expression of IL-12p40 and suppressed IL-10, and these M1 macrophages were capable of potentiating Th1 and Th17 responses[227]. Specific depletion of suppressor of cytokine signalling 3 (SOCS3) in the myeloid lineage of mice to induce a strong M1 macrophage bias was associated with an enhanced phagocytic capacity[228]. Notch receptor signalling through recombination signal binding protein for immunoglobulin kappa J region (RBPJ) modulates production of IRF8 which then elicits expression of M1 macrophage genes including IL-12 and NOS2 (inducible nitric oxide synthase)[229]. Peroxisome proliferator-activated receptor-γ (PPARγ) is a nuclear receptor commonly known for its expression in adipose tissue and its roles in the regulation of adipocyte differentiation and glucose homeostasis[230, 231]. PPARγ is also upregulated in activated macrophages where it inhibits the signalling of the transcription factors activator protein (AP-1), signal transducer and activator of transcription 1 (STAT1) and NF-κB[232]. It is through such functions that PPARγ is able to regulate inflammatory responses. This transcription factor was shown to induce M2 differentiation in primary human monocytes which was associated with enhanced anti-inflammatory activity[233]. NF-κB is of course involved in M1 polarisation, but p50, a subunit of the NF-κB complex, is inhibitory to NF-κB function and is thereby involved in M2-type responses[234]. Krüppel-like factor 4 (KLF4) is strongly expressed in M2 macrophages and interacts with STAT6 to initiate a M2 genetic program. Accordingly, KLF-4 deficient macrophages showed enhanced M1 function through amplified pro-inflammatory and anti-bacterial activity[235]. Many other factors have been associated with M2 polarisation including cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), Jumonji domain containing-3 (Jmjd3) and cMyc[236-238]. The abundance of factors regulating macrophage polarisation is unsurprising given the vast number of inciting stimuli that can influence macrophage phenotype and function and consequently the multitude of activation states that macrophages can occupy.

*Metabolism*

Another element which differs between macrophage phenotypes is their metabolic tendencies. One such differentiation can be observed in the glucose metabolism of these subsets. Macrophages polarised to IFNγ or TLR ligands advocate anaerobic respiration through the glycolytic pathway with the aim of facilitating bactericidal activity in hypoxic environments whereas alternatively activated macrophages showed no substantial shift in glycolytic processing[239]. Rather the M2 macrophage employs the use of fatty acid oxidation and oxidative glucose metabolism which is sustained for longer time periods and this is essential for their functions in tissue remodelling and repair which are lengthy and highly energy-dependent processes[240]. Lipid metabolism is another distinguishing factor in macrophage polarisation, unsurprising considering the prominent role of PPARγ in both regulating macrophage phenotype and in lipid metabolism[241]. Martinez *et al* performed transcriptome analysis of human MDMs (hMDMs) and highlighted the increased expression of cyclooxygenase-2 (COX-2) along with downregulation of COX-1 and arachidonate 5-lipoxygenase in M1 macrophages. M2 cells had increased expression of COX-1 and the M2 marker 15-lipoxygenase[242]. The COX enzymes are responsible for production of prostaglandin synthesis, an important anti-inflammatory mediator[243]. This study also showed the differential regulation of sphingolipid mediators including sphingosine 1-phosphate and ceramide 1-phosphate. There is evidence suggesting an anti-inflammatory role for sphingosine 1-phosphate in murine macrophages where the lipid mediator was able to reduce TNFα production and increase arginase-1 activity[244]. Ceramide 1-phosphate has been shown to promote survival and the proliferation of macrophages[245, 246]. As mentioned earlier M2 macrophages utilise fatty acid oxidation for respiration and the energy produced is important for their function. It has also been observed that saturated and unsaturated fatty acids will differentially affect macrophage phagocytosis, with polyunsaturated fatty acid supplemented macrophages capable of more phagocytosis[247]. The amino acid L-arginine is handled by iNOS in M1 macrophages to produce nitric oxide, which is an important antimicrobial effector[248, 249]. Nitric oxide also has a broad impact on immune responses of other cells, targeting T cells and NK cells for example[250, 251]. Moreover, mice deficient in iNOS presented aberrant immune responses characterised by an amplified Th1 response, susceptibility to parasitic infection and resistance to LPS-

induced mortality[252]. M2 mouse macrophages however utilise arginase-1, which is regulated by STAT6, to convert L-arginine to L-ornithine[253]. L-ornithine represents a precursor for L-proline which modulates collagen production, an important element of tissue remodelling[254]. One of the homeostatic functions of macrophages involves modulating the availability of iron. Human macrophages stimulated with LPS and IFNγ suppression in ferroportin (an iron exporter) expression and amplified H ferritin (an iron storage protein) expression leading to iron sequestration. Conversely, differentiation in M2-promoting IL-4 resulted in the opposite effect and increased extracellular iron levels[255]. The aim in M1 macrophage iron retention is to deplete the environment of this micronutrient which is essential for bacterial growth. The M2 macrophages efflux of iron can result in enhanced cell proliferation and collagen synthesis thereby facilitating tissue remodelling[256, 257].

*Murine vs. human macrophages*

A majority of work in the field of macrophage polarisation was produced by investigating murine macrophages. It is increasingly apparent however that there are significant differences in human and murine macrophages with regards to characterisation of the various phenotypes. For example, mouse M1 macrophages are known to express NOS, however this is not true of human M1 macrophages[258]. Mouse M2 macrophages are often identified using Arginase-1 and Ym1 markers, however these are not expressed on human alternatively activated macrophages[259]. One of the few markers which are consistent between the two species is transglutaminase-2, expressed by both human and mouse M2 macrophages[260]. A number of groups have focused their attentions on characterising human macrophages under the influence of different polarising stimuli. Tarique *et al* found that hMDMs exposed to M1 stimuli LPS and IFNγ consistently expressed CD64 and CD80, whereas IL-4/IL-13 treated M2 MDMs were identified by CD11b and CD209[261]. The markers CD163 and CD206 are considered robust markers for the identification of human M2 macrophages and have been used in a number of studies[233, 262-265]. With the ever expanding diversity of macrophage populations, there is a growing need to create a standardised nomenclature and definition process. Secondly, the methods employed by different groups in the culture methods for macrophages can have

profound effects on macrophage phenotype and function making comparisons difficult. A collaborative work in 2014 addressed this issue and proposed a set of guidelines for the field encouraging transparent and detailed publication of culture techniques and the labelling of macrophage phenotypes with the inciting stimuli e.g. M(IL-10)[266]. This will hopefully facilitate consensus in an area of macrophage biology which is currently lacking in it.

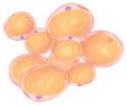
### 1.3.3 The alveolar macrophage (AM) in lung injury

The importance of macrophage polarisation and function in disease is becoming increasingly apparent. Whilst macrophages are essential for many normal physiological functions and homeostasis, in aberrant conditions they contribute to disease pathophysiology **(Fig 1.3.3)**. The classically activated macrophage is essential for the clearance of pathogens but there is often extensive collateral damage to the surrounding tissue as a result of reactive oxygen and nitrogen species production[267, 268]. If the pro-inflammatory state of these macrophages is not tightly regulated this can lead to a number of chronic inflammatory disorders such as atherosclerosis, multiple sclerosis and chronic venous leg ulcers[269-271]. Conversely, a type of alternatively activated macrophage described as tumour associated macrophages have been shown to enhance tumour growth and metastasis, through mechanisms such as angiogenesis[272].

Macrophage polarisation also plays a role in lung injury. Compared with other groups, AMs from patients of ARDS have been shown to have increased expression of 27E10, a marker associated with acute phase monocytes and macrophages, along with decreased RM3/1 which is linked to dampening inflammation. This suggests a skew towards a more pro-inflammatory M1 phenotype[273]. In the same study by Rosseau *et al.,* it was observed that continued expression of M1 markers as well as increased levels of MCP-1, a key pro-inflammatory cytokine produced by these macrophages, was linked to a poorer prognosis. Furthermore, production of M1 cytokines IL-1, IL-1β and IL-8 by AMs of patients of ARDS was higher than that of healthy controls and the higher IL-8 levels were linked to increased mortality of these patients[274, 275]. In humans, study of the potential role of AMs in the resolution phase of ARDS is limited.

It has been observed that in septic patients of ARDS a continuing increase in neutrophil numbers accompanied by a decrease in AMs in the BALF of these patients at days 7 and 14 portended a poorer outcome[276]. It could be speculated that AMs enhance resolution through mechanisms such as efferocytosis of apoptotic neutrophils leading to the production of anti-inflammatory TGFβ and lipoxins[277-279].

*Fig 1.3.3: Macrophages in development, homeostasis and disease.*

| Normal physiology | | Pathology |
|---|---|---|
| Microglia, (neuronal patterning, fluid balance) |  | Neurodegeneration |
| Osteoclasts and macrophages (bone remodelling; haematopoiesis) |  | Osteoporosis and osteopetrosis Leukemia |
| Heart and vasculature |  | Atherosclerosis |
| Kupffer cells (lipid metabolism, toxin removal) |  | Fibrosis |
| Branching morphogenesis |  | Cancer and metastasis |
| Metabolism; adipogenesis |  | Obesity and diabetes |
| Immunity |  | Arthritis, EAE, IBD |

*Wynn T. et al, 2013[280]*

While human data are limited, there is an abundance of evidence defining the AM as a key player in the pathophysiology of ARDS from injury to resolution. In an ischaemia-reperfusion model of lung injury, depletion of AMs using clodronate-containing liposomes resulted in decreased pulmonary vascular leakage, TNFα, MCP-1 and MIP-2[281]. Depletion of AMs in a model of ventilator-induced lung injury reduced development of pulmonary oedema[282]. These studies outline the heavy involvement of AMs in the initiation of lung injury. Another study demonstrates

however that the removal of AMs triggers an over three-fold increase in neutrophilia after endotoxin-induced lung injury. This suppressive effect on recruitment appeared to result from AM inhibition of alveolar epithelial cell monocyte chemoattractant protein-1 production[283]. A similar study shows that without AMs, mice succumb to *Klebsiella pneumoniae (K. pneumoniae)* infection with no survival after 3 days. Intriguingly, this was associated with much greater neutrophil influx and levels of TNFα and macrophage inflammatory protein-2[284]. While all of these studies confirm that AMs are important in lung injury pathology, the effects of their absence seem to vary and suggest both promotion of inflammation but also its regulation. The discrepancies here are possibly due to the differences in the models being investigated; in non-infectious models, depletion of AMs mitigates injury whereas they are required to stifle excessive neutrophil recruitment and promote effective bacterial clearance in infectious models of lung injury. As for resolution, a study assessing pulmonary macrophage populations in *Pseudomonas aeruginosa (P. aeruginosa)*-induced lung injury demonstrates an association between M1 macrophages in the initial stages of injury but increased prevalence of M2 macrophages during the resolution phase[285]. This would suggest that the M2 macrophage plays a role in the resolution of injury and that promotion of an M2 AM profile therapeutically may reduce injury and improve outcomes.

### 1.3.4 Interactions of MSCs with macrophages

As described earlier, MSCs possess extensive immunomodulatory capacity and influence many kinds of immune cell. MSCs and macrophages interact very closely and through these interactions MSCs have a strong influence on the inflammatory balance in these tissues. Their ability to induce T regulatory cells was shown to be partly mediated by the promotion of monocyte derived-M2 type macrophages characterised by CD206 and CD163 expression[58]. MSCs have been shown to modulate macrophages in a cecal ligation and puncture of model of sepsis to increase their IL-10 production through PGE2 production. Moreover, the depletion of macrophages in this model diminished the beneficial effects of MSCs, suggesting that macrophages are cellular mediators of the MSC effect *in vivo*[85]. Maggini *et al* described the ability of murine MSCs to inhibit TNFα and IL-6 while increasing IL-

10 production by LPS treated peritoneal macrophages[286]. Corroborating these findings with human cells, Kim and Hematti describe "MSC-educated macrophages" which exhibit increased CD206 expression, high IL-10 and IL-6 but low IL-12 and TNFα production[287].

Concentrated culture medium from bone marrow MSCs was able to increase recruitment of macrophages to wounds in mice and was associated with accelerated wound healing[288]; wound repair is a function associated with alternatively activated macrophages. These findings are further supported with a myocardial infarction model where human MSCs were able to reduce total monocyte/macrophage counts whilst increasing the proportion of M2 macrophages present. MSC treatment resulted in enhanced fractional shortening 2 weeks after myocardial infarction[289]. hMSCs which had formed spheroid aggregates produced higher levels of PGE2 which resulted in more potent induction of an M2-macrophage phenotype defined by increased IL-10 and IL-1ra secretion and expression of the M2 marker CD206 (mannose receptor)[290]. The MSC regulatory effect on macrophages is also mediated by microvesicles containing miRNAs which are known to suppress TLR signalling and macrophage activation. This was necessary for the acceptance of mitochondria from the MSCs and subsequent enhancement in bioenergetics of the macrophage[291]. MSCs have also been shown to modulate AMs; MSCs or their culture medium were able to increase arginase activity (M2) and decreased iNOS expression (M1) in AMs of LPS-injured mice. MSCs in this model reduced lung injury which was partly mediated by insulin-like growth factor-1[292]. Use of adipose, umbilical cord or bone marrow-derived MSCs resulted in increased alveolar macrophage numbers in a mouse model of allergic asthma and diminished key features of asthma including hyperresponsiveness and eosinophilic infiltration. Artificial removal of the macrophage compartment using clodronate-containing lysosomes reversed the beneficial effect of MSCs[293]. It is clear that MSCs communicate with many cells in their environment, not least the macrophage, and that this provides a means by which they can modulate inflammation.

## 1.4 Extracellular vesicles (EVs)

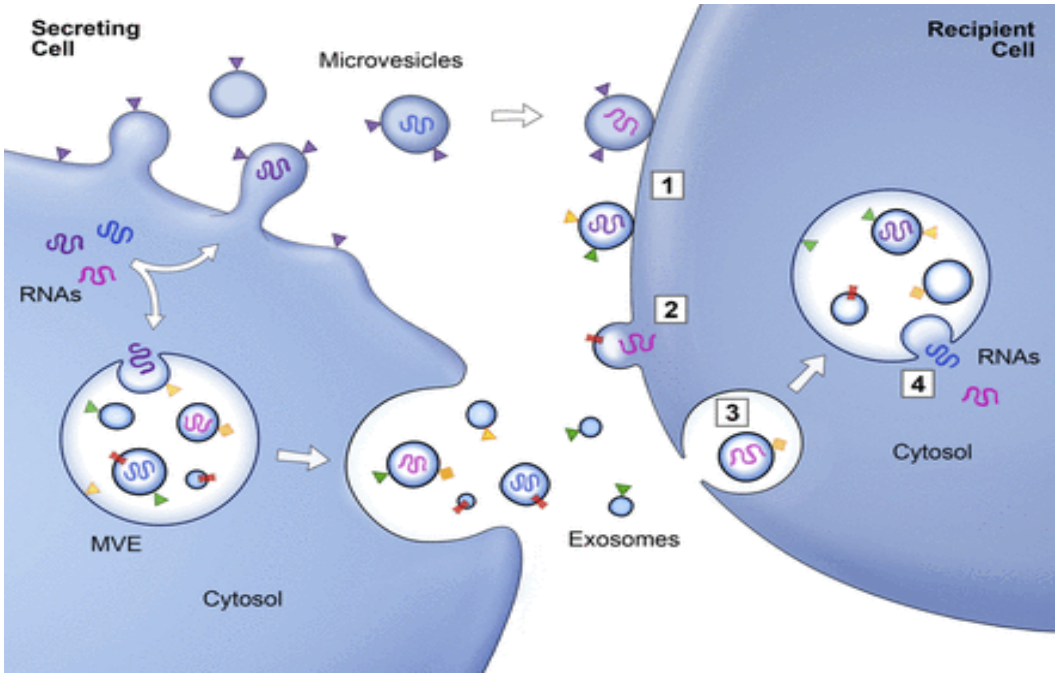
### 1.4.1 Introduction

Initially cell-cell contact and the release of soluble mediators were thought to be the sole means of cellular communication. More recently an additional mode of communication has been identified, the transfer of extracellular vesicles. EVs were first described by Trams *et al* in 1981[294]. These membrane-bound compartments may carry a plethora of different cargos from proteins to different species of RNA[295]. Secretion of EVs is known to regulate many important biological functions including sperm motility, antigen presentation and promoting tumour progression[295-297]. EVs may be subdivided into two groups based on their size; EVs ranging from approximately 40-100nm in diameter are termed exosomes and larger EVs of up to 1000nm are often called microvesicles (MVs)[298]. However the distinction between these two classes is often difficult given that size, morphology and protein composition often fail to separate them[299].

EV secretion has been reported in virtually all cell types including adipocytes, endothelial progenitor cells, macrophages and microglia and is also present in bodily fluids such as blood, saliva and cerebrospinal fluid[300-306]. EVs are secreted by two key pathways **(Fig 1.4.1)**; fusion of multivesicular endosomes with the plasma membrane and direct budding from the plasma membrane[307, 308]. EVs will target neighbouring cells and are received either through fusion of the EV membrane with the cell membrane of the target cell or through endocytosis[309, 310]. It is apparent that transfer of EVs between cells has an element of specificity, for example B lymphocyte exosomes solely bind dendritic cells of the lymphoid follicles and EVs from intestinal epithelial cells interact more efficiently with dendritic cells than with lymphocytes[311, 312]. Whilst the mechanisms regulating EV-cell interactions are not fully understood, a number of conditional and cell-specific elements are emerging. Such evidence is provided by the observation that T cells selectively bind MHC class II-containing exosomes from dendritic cells produced after interactions between these two cell types[313]. This recruitment was dependent on induction of an altered state of leukocyte function-associated antigen-1 (LFA-1) brought about by T cell activation[314]. Unsurprisingly, it seems that the EV membrane protein profile modulates cell

targeting. Tetraspanins are a family of small transmembrane proteins which form intricate and complex interactions with a broad range of integral proteins and the expression of certain tetraspanins on the EV surface influences their targeting[315]. For example, tetraspanin-8-expressing exosomes preferentially bind endothelial and pancreatic cells[316].

*Figure 1.4.1: Transfer of EVs*




*Raposo G. and Stoorvogel W., 2013[298]*

### 1.4.2 MSC-derived EVs

As mentioned earlier, MSCs also produce EVs. The role of MSC-derived EVs in their therapeutic effects is a relatively recent addition to their paracrine repertoire. Indeed, the potential therapeutic effect of MSC-EVs is being investigated in a range of preclinical disease models. The systematic review referenced earlier by Akyurekli *et al* focuses on a range of these studies in the context of acute kidney injury, liver injury and myocardial infarction amongst others[175]. In glycerol-induced acute kidney injury, MSC-EVs were found to be therapeutic to a similar extent as the cells themselves with reduction in tubular necrosis, lesion size and blood urea nitrogen levels[167]. MSC-EVs have been shown to inhibit tumour progression *in vivo* and induce cell death in a number of tumour cell lines *in vitro*[166]. MSC-derived exosomes were also capable of

reducing infarct size following myocardial ischaemia/reperfusion injury in mice[169]. Moreover, Cruz *et al* were able to demonstrate that MSC-EVs mirror the beneficial effects of MSCs in their *Aspergillus* hyphal extract-induced airway inflammation model[168]. In the context of lung disease, MSC exosomes were found to reduce pulmonary hypertension through the suppression of hypoxia-induced STAT3 expression[170]. MSC-derived EVs alone were capable of attenuating *E. coli*-induced lung injury in mice and recapitulating many of the therapeutic effects of the cells themselves, including decreases in pulmonary oedema and inflammation. mRNA coding KGF contained within these vesicles was partially responsible for this phenomenon[317]. Recently, Monsel and colleagues demonstrated that MSC-EVs were taken up by human alveolar epithelial cells and monocytes, which was dependent on the CD44 receptor. Monocytes which had received EVs demonstrated enhanced phagocytosis[171]. Interestingly, *in vivo* imaging depicts the formation of connexin-43 based gap junctions between MSCs and alveolar epithelial cells allowing the transport of mitochondria to the epithelia via EVs. The resultant increase in adenosine triphosphate (ATP) levels concomitantly resulted in restoration of surfactant secretion by type II pneumocytes, reduced alveolar permeability and mortality in a lipopolysaccharide (LPS) injury model[318].

Whilst it is well established that many of the benefits of MSC paracrine factors are due to EVs, work is ongoing in discerning the exact contents of MSC-EVs and the functional aspects of these contents. EVs contain a range of different proteins but there has been a particular interest in the extensive microRNA content of MSC-EVs. miRNA screening of bone marrow MSC-EVs highlighted a multitude of miRNAs which are associated with vital cell processes such as cell survival and differentiation, organ development and in immune regulation[319]. MSC-derived exosomes were capable of transferring miR-133b to neurones and astrocytes and promoting neurite outgrowth[320]. A recent paper attributes pro-angiogenic properties to adipose-derived stem cells; it was observed that treatment of these stem cells with medium used to promote endothelial differentiation enhanced their angiogenic effects towards venous endothelial cells *in vitro* and murine aortic rings *ex vivo*[321]. EV-derived miR-31 which targets the anti-angiogenic protein, factor-inhibiting hypoxia-inducible factor-1 (HIF-1), was responsible for these effects. An elegant study by Phinney *et al* highlights

MSC export of depolarised mitochondria via EVs to human macrophages which enhances their bioenergetics whilst alleviating oxidative stress from the MSCs. The exosome fraction containing miRNAs resulted in the suppression of toll-like receptor (TLR) signalling by macrophages which facilitates the uptake of the foreign mitochondria[291]. In the same study, this group also observed differential miRNA profiles in the MSC exosomes and their cells of origin. For example, miR-451a and miR-1202 were present at 316 and 45-fold higher levels respectively in exosomes compared to MSCs. Conversely, miR-125b levels were 148-fold higher in the cells compared to exosomes. This suggests that there are mechanisms in play which actively recruit specific miRNAs to exosomes for their export. Accumulating evidence on the benefits of MSC-EVs in preclinical models not only sheds light on mechanisms of MSC effect but also raises the possibility of the use of isolated EVs in place of the cell therapy. This has the potential advantage of reducing the risk of erroneous differentiation of engrafted cells or the development of MSC-derived tumours. Although this is not to say that EVs have no oncogenic tendencies, and this should also be investigated. A phase I clinical trial in stage III/IV melanoma patients using exosomes isolated from dendritic cell cultures showcased the feasibility of large-scale production of clinical grade exosomes for administration to patients[322]. There are currently a number of trials registered investigating the effects of EVs in patients, including the role of MSC-EVs in type I diabetes mellitus (registered on clinicaltrials.gov, identifier: NCT02138331).

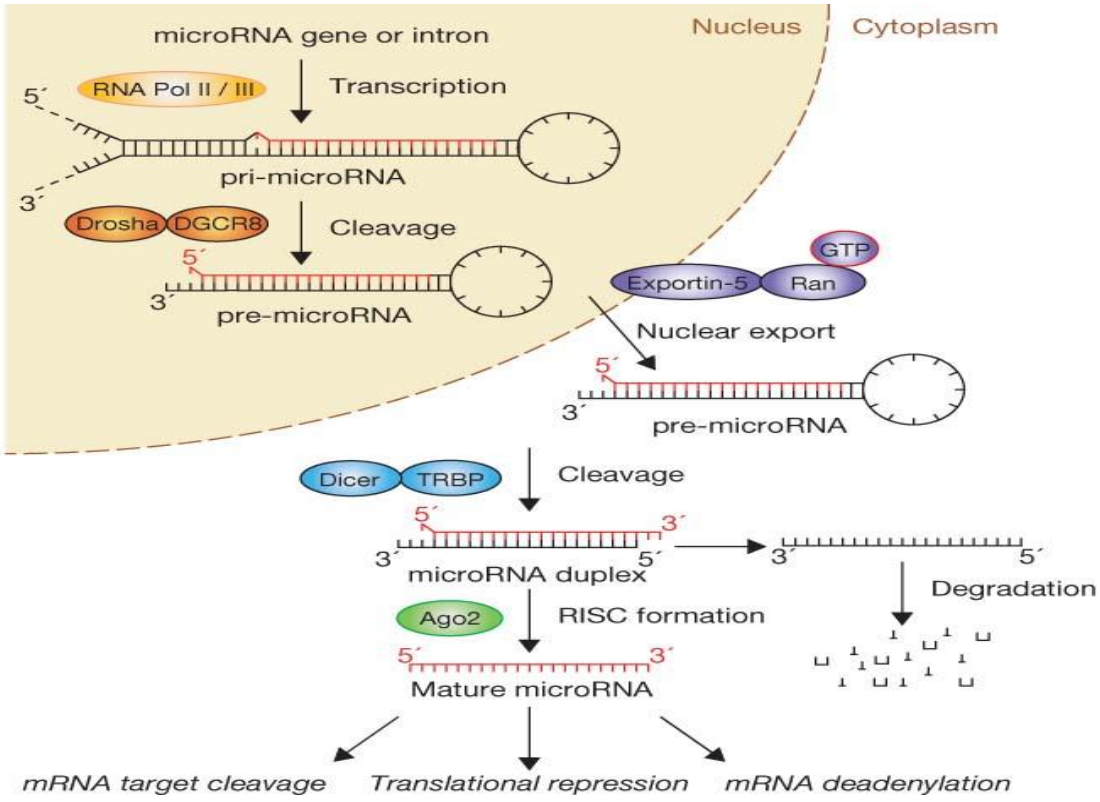
## 1.5 MicroRNA (miRNA)

### 1.5.1 Introduction

miRNA are a species of RNA which are single-stranded and little over 20 nucleotides in length and are essentially regulators of gene expression at the post-transcriptional stage[323]. The miRNA biogenesis process is outlined in **Figure 1.5.1**. miRNAs are transcribed by RNA polymerase II and III to form the primary miRNA (pri-miRNA) transcript, which consists of a hairpin stem attached to two single strands and a terminal loop[324]. This transcript is processed by the Drosha-DiGeorge critical region 8 (DGCR8) complex within the nucleus resulting in cleavage of the hairpin stem, removing sections of both the 3' and 5' strands and producing precursor miRNA (pre-

miRNA)[325]. Pre-miRNA is then transported to the cytoplasm by the Exportin-5/Ran-GTP complex where the RNase III enzyme Dicer cleaves the terminal loop from the pre-miRNA leaving a double-stranded miRNA structure or duplex[326, 327]. This duplex consists of a guide strand and a passenger strand which are separated by the activity of helicases including p68 and RNA helicase A[328, 329]. The passenger strand is subject to degradation while the functional guide strand may then be incorporated into the RNA-induced silencing complex (RISC) which is facilitated by Argonaute2[330]. When bound to a guide strand, the RISC complex will bind to specific mRNA targets of the miRNA based on complementary base pairing. The RISC complex may then inhibit translation by degrading the mRNA or preventing translational initiation or elongation[331, 332].

*Figure 1.5.1: The miRNA biosynthesis pathway*




*Winter J. et al, 2009[324]*

Unlike small interfering RNA (siRNA), miRNA does not require absolute complementary base pairing with its target mRNA and this helps explain the capacity of one miRNA to bind multiple mRNA targets[333]. With the diversity of miRNAs and

the promiscuity of their function, it is no surprise that they have been implicated in cellular processes including differentiation, proliferation and metabolism[334, 335]. Then of course miRNAs also play roles in disease such as cancer and multiple sclerosis[336, 337].

### 1.5.2 miRNAs and macrophages

Macrophage function and phenotype are heavily influenced by miRNAs. Firstly, the maturation of monocytes to macrophages is regulated partially by miR-155, miR-222, miR-424 and miR-503. miR-155 promotes cessation of the cell cycle by hindering progression of the G2 phase of the cell cycle and miR-222 promotes apoptosis. miR-424 and miR-503 repress cell cycle regulators Cyclin D1 and Cyclin E1 thereby inhibiting the G1 phase. Furthermore, miR-424 and miR-503 both target the transcript sequence of miR-9-3 which itself has anti-differentiation properties[338]. A plethora of miRNAs have emerged which modulate macrophage activation in inflammatory responses and other functions. miR-155 is upregulated robustly in macrophages in response to IFN-β and the TLR3 ligand polyriboinosinic:polyribocytidylic acid (poly(I:C))[339]. miR-155 is also induced by LPS and enhances TNFα production while miR-125b is concomitantly suppressed. miR-125b binds the 3' untranslated region of TNFα mRNA preventing its translation and so its suppression is an important requirement for proper TNFα production in these inflammatory responses[340]. A homologue of miR-125b, miR-125a-5p, decreases macrophage production of other inflammatory cytokines in response to oxidised low density lipoprotein including IL-6, TNFα and TGFβ[341]. The treatment of human mononuclear cells from peripheral blood with LPS induces expression of miR-21 through MyD88 and NF-κB. miR-21 is a negative regulator of TLR4 signalling and acts through targeting the tumour suppressor and pro-inflammatory protein programmed cell death 4 (PDCD4). This results in increased IL-10 secretion and lesser activation of NF-κB[342]. Inflammatory responses from innate immune cells must be tightly regulated to prevent excessive collateral tissue damage; whilst some miRNAs are involved in promoting the inflammatory responses, others may provide a negative feedback effect. One such miRNA is miR-146a, induced by *S.pneumoniae* in macrophages, which acts to suppress interleukin-1 receptor-associated kinase 1 (IRAK1) and TRAF6 of the TLR2

pathway and also COX2 and IL-1β, two key inflammatory mediators[343]. Phagocytic capacity of macrophages may also be affected by miRNAs, as described by Jiang *et al*[344]. miR-615-3p in splenic macrophages targets ligand-dependent nuclear receptor corepressor (LCoR) and its downregulation allows unmitigated PPARγ expression to occur. PPARγ is then responsible for enhancing phagocytic activity. A recent study emphasises the importance of miRNA in regulating macrophage autophagy but also the ability of pathogens to exploit miRNA function for their own benefit. *Mycobacterium tuberculosis (M. tuberculosis)* is able to survive when internalised by macrophages and achieves this through promoting miR-33 expression. This miRNA blocks autophagy by targeting mediators like autophagy-related gene 5 (ATG5) and lysosome-associated membrane protein (LAMP1) but also inhibiting forkhead box O3 (FOXO3) and transcription factor EB (TFEB) allows lipid accumulation providing nourishment and fuelling *M. tuberculosis* replication[345]. Macrophage polarisation results from a substantial shift in the gene expression profile, as does any change in cell phenotype.

Macrophage phenotype may also be augmented at the post-transcriptional level by miRNAs. Consistent with the enhanced TNFα production with miR-155, this miRNA has also been demonstrated to promote polarisation of M2 tumour-associated macrophages towards the pro-inflammatory M1 phenotype. Macrophages driven towards the M1 phenotype by overexpressing miR-155 exhibited anti-tumour properties[346]. Conversely, miR-223 is a regulator which induces an alternatively activated macrophage phenotype, evidenced by increased expression in IL-1β, IL-6 and TNFα but reduced PPARγ and Arginase 1 in bone marrow-derived macrophages deficient in miR-223[347]. LPS stimulation of murine macrophages reduces miR34a levels, another negative regulator of TLR signalling which binds Notch1[348]. Interestingly, while the TNFα suppression function of miR-125b would imply anti-inflammatory activity, this miRNA has also been shown to amplify and extend NF-κB signalling and so promoting pro-inflammatory functions. This is achieved through the inhibition of a negative regulator of NF-κB, tumour necrosis factor alpha-induced protein 3 (TNFAIP3), and reducing IRF4 expression[349, 350]. Macrophages with high levels of miR-125b are attributed with exaggerated antigen presentation capacity and so more efficiently stimulate T cells.

### 1.5.3 miRNAs and MSCs

MSC express a multitude of miRNAs and a number of studies have screened MSCs and their MVs for their miRNA profiles[291, 319, 351]. miRNAs responsible for MV-mediated MSC beneficial effects in preclinical models were discussed in *Section 1.4.2* and will not be repeated. miRNAs, like in the macrophage, influence MSC function and phenotype and also facilitate stem cell activities. miR-145 provides an inhibitory signal on chondrogenic differentiation of MSCs by targeting SRY-related high mobility group-Box gene 9 (Sox9). Downregulation of miR-145 is necessary for efficient differentiation into chondrocytes and this can be accomplished with TGFβ3 stimulation[352]. During osteogenic differentiation of MSCs, miR-20a is increased and using mimic miR-20a promoted their differentiation by induction of BMP/Runx2 signalling[353]. This miRNA targets PPARγ, an inhibitor of BMP/Runx2 signalling. Proliferation and immunoregulatory capacity of MSCs may be partly diminished by increased miR-181a levels, which occurs in severe preeclampsia. miR-181a transfected MSCs exhibited increased IL-6 expression with more pronounced activation of the p38 and JNK pathways[354]. miR-27b targets SDF-1 and inhibits its production by mouse MSCs and this was associated with attenuated migration of MSCs to burn wounds of the epidermis in mice and subsequently abrogated wound repair[355]. MSCs have been reported to promote cancer cell invasion and metastasis[54, 356]. The miRNA let-7 is downregulated in MSCs associated with prostate cancer which alleviates inhibition of IL-6 expression by these cells. IL-6 production was responsible for the pro-tumour effects of these MSCs[357].

## 1.6 Mitochondria

Mitochondria are a membranous organelle found ubiquitously in eukaryotic cells and are best known for their role in oxidative respiration and as the 'powerhouse' of the cell[358]. In the presence of oxygen, the electron transport chain, situated in the inner membrane of mitochondria, facilitates the active pumping of protons into the intermembrane space. The resultant proton gradient is utilised by ATP synthase to phosphorylate adenosine diphosphate (ADP) to ATP. Apart from respiration, mitochondria perform other essential cellular functions. Mitochondria can mediate apoptosis via the intrinsic pathway through the release of cytochrome c[359].

Cytochrome c contributes to the formation of the apoptosome which activates caspase 9 and this is turn initiates the death machinery. Mitochondria are an important source of reactive oxygen species including superoxide and nitric oxide[360]. Reactive oxygen species (ROS), produced during respiratory burst in macrophages and neutrophils, are known to facilitate bacterial killing[361]. Moreover, ROS have been shown to promote production of pro-inflammatory cytokines in response to LPS[362]. Mitochondrial ROS production in macrophages is enhanced when the cells receive signals through TLR1, 2 and 4. This TLR signalling gathers mitochondria towards phagosomes where the TLR signalling molecule TNF receptor-associated factor 6 (TRAF6) interacts with evolutionarily conserved signalling intermediate in Toll pathways (ECSIT) and augments ROS production[363]. Interestingly though, the inhibition of mitochondrial function through the use of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (uncouples oxidative phosphorylation) or oligomycin (a $F_0$ ATPase inhibitor) had no effect on phagocytic capacity in human neutrophils[364]. However FCCP was able to inhibit chemotaxis and prolonged oligomycin treatment hindered the initiation of respiratory burst.

The role of mitochondria in innate immune functions goes beyond ROS generation. Mitochondria are integral to TLR-independent responses to viral infection. Viral RNA is sensed by cytosolic helicases known as retinoic acid-inducible gene-1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA-5)[365, 366]. These then activate mitochondrial antiviral-signalling protein (MAVS) found on the outer membrane of mitochondria which recruits TRAF-3 and TRAF-6 ultimately leading to IRF-3 and IRF-7 activation for transcription of type I IFN genes as well as NF-κB for expression of yet more pro-inflammatory mediators[367-370]. Mitochondrial ROS does not only contribute to phagocytic respiratory burst and killing of intracellular microbes but has been linked with activation of the NLRP3 inflammasome[371]. Given mitochondrial remnants from necrotic cells, macrophages would respond with caspase-1 and IL-1β production, two key functions of the NLRP3 inflammasome[372]. Moreover, when autophagy, the process responsible for normal organelle turnover, is inhibited, dysfunctional mitochondria will accumulate and produce ROS in abundance. This is accompanied with amplified IL-1β and IL-18 responses after LPS and ATP-induced inflammasome activation[373]. There is a long-standing theory that mitochondrial

function is a major contributor to the natural aging process[374]. They are major producers of free radicals which are believed to inflict oxidative damage to cells throughout our lifetime[375, 376]. This damage includes DNA strand breakage, the cross-linking of DNA to protein, as well as protein to protein, and lipid oxidation. Mitochondria themselves will become altered with age both morphologically and functionally. It has been observed that in aging rat neurones their mitochondria develop abnormal filamentous bodies and glycogen deposits altering their inner matix[377]. Similarly, in aged human skeletal muscle, there are reports of enlarged mitochondria which have disorganised and discontinuous cristae[378]. The mitochondria of aged cells have been shown to exhibit decreased activity in complexes I and IV of the electron transport chain whereas complexes II and III are more or less consistent with that of younger mitochondria. This is interesting given that mitochondrial DNA encodes complexes I and IV and complexes II and III are derived from nuclear DNA[379].

Further support is afforded to mitochondrial implication in the aging theory with the observation that patients of human immunodeficiency virus (HIV) treated with anti-retroviral drugs age more prematurely. Payne *et al* show that patients who had received this treatment had increased prevalence of defective cytochrome c oxidase-succinate dehydrogenase in skeletal muscle compared to healthy controls or even HIV patients not receiving this treatment. They then went on to demonstrate that cells with these defects had increased total mitochondrial DNA content compared to normal cells of the same tissue sample. This would suggest that this phenomenon is a result of increased mitochondrial DNA propagation rather than increased occurrence of mutagenesis[380]. The premature aging offers an explanation for the high prevalence of cardiovascular and neurological complications reported in these patients, unsurprising since cardiomyocytes and neurones are examples of post-mitotic cells and so are more susceptible to functional decline[381, 382]. As mentioned earlier, MSCs are capable of mitochondrial transfer through the use of TNTs or microvesicles[116, 117, 291, 318]. This mitochondrial donation can have profound effects on the recipient cells including the enhancement of bioenergetics and phagocytic capacity[116, 291, 318]. The enhancement in phagocytosis by human macrophages demonstrated by our group through TNT-mediated mitochondrial transfer could be explained simply by the improved

bioenergetic profile. It is feasible that a larger supply of ATP could be utilised by these macrophages for phagocytosis which requires extensive cytoskeletal rearrangement and is a highly energy-dependent process[383, 384]. It is already understood that mitochondrial propagation and function are dependent on nuclear gene expression[385]. In fact, many of the proteins required for the oxidative phosphorylation process are encoded in the nuclear DNA. Nuclear respiratory factors (NRF), NRF1 and NRF2 are important activators of mitochondrial transcription factor A, a key promotor of mitochondrial gene expression, for example[386]. But it appears that this interplay is bidirectional and that mitochondria may also influence the function and phenotype of the cell that it resides in. Indeed, mitochondrial transfer appears to have influence beyond metabolic dynamics as evidenced by Acquistapace *et al* who demonstrated that mitochondrial transfer from adipose tissue MSCs to mature cardiomyocytes resulted in their dedifferentiation towards a progenitor-like phenotype[387]. This effect was associated with "partial" fusion of MSCs with the cardiomyocytes and the transfer of MSC mitochondria to these myocytes. Another study highlights the importance of proper mitochondrial function in the ability of embryonic stem cells (ESCs) to differentiate. This was shown when the uncoupling of oxidative phosphorylation in mitochondria resulted in diminished differentiation capacity which was associated with aberrant Hox gene expression in ESCs[388]. Mitochondrial DNA mutations are also strongly associated with cancer progression, including prostate cancer. Mutations in the cytochrome oxidase subunit 1 gene were found to be prevalent in patients of prostate cancer. Moreover, the artificial introduction of this mutant gene into a prostate cancer cell line resulted in 7-fold accelerated tumour growth when introduced into mice compared to the untreated cancer cells[389].

## 1.7 Aims of the study

ARDS is a devastating clinical disorder in ICUs worldwide which currently has no effective therapy available. ARDS arises from a multitude of aetiologies and has a complex pathophysiology which may explain the difficulty experienced thus far in developing an effective intervention. AMs are central to the orchestration of inflammatory responses in the lung including that of patients of ARDS[275, 281-283, 285, 390]. A therapy capable of modulating alveolar macrophage function could therefore

help to dampen inflammatory responses thereby reducing the extent of lung injury and perhaps improving outcomes. MSCs represent a promising candidate cellular therapy for patients of ARDS. Supported by an abundance of literature reporting their beneficial effects in preclinical models of lung injury, MSCs have progressed to clinical trials in these patients. Several of their mechanisms of effect, which appear to be paracrine in origin, have been elucidated but their modes of action have not been fully investigated yet. It is imperative that any given therapy is thoroughly understood for their safe, appropriate and effective application in the clinic. The study of MSCs in the context of lung injury to date has heavily favoured the use of mouse models and whilst this is crucial for the determination of an effect *in vivo*, its relevance to human patients is limited. Research of hMSCs in physiologically relevant models, using human cells and samples of ARDS patients will provide invaluable insight into how these cells may behave as a cellular therapy. Moreover, mechanistic studies of these interactions may facilitate more effective therapeutic application through the development of potency assays and may even identify further therapeutic targets. Potency assays are of particular importance in cellular therapy given the heterogeneity that exists between donor cells. Reliable readouts which enable selection and then propagation of a quality, clinical grade, off-the-shelf cell therapy is a necessity.

Given that MSCs are renowned for their immunomodulatory tendencies, it was hypothesised that:

*Human MSCs will promote an anti-inflammatory M2-like phenotype in human MDMs through paracrine mechanisms.*

The aims of this project are therefore to:

- Determine the effect of hMSCs on hMDM function and phenotype
- Elucidate the mechanisms of these effects.

# Chapter 2

# Materials and methods

## 2.1 Human MSC culture

### 2.1.1 Culture of human bone marrow-derived MSCs

Bone marrow-derived hMSCs were provided by the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine at Scott & White through a grant from NCRR of the National Institute of Health (NIH), Grant # P40RR017447. These hMSCs were extensively characterised by the supplier testing for plastic adherence, expression of key mesenchymal markers and absence of haematopoietic markers, as well as differentiation capacity for a number of mesenchymal lineages. These assessments fulfil the criteria put forward by the International Society for Cellular Therapy for characterisation and identification of MSCs. MSCs were cultured under standard tissue culture conditions; 37ºC, 5% $CO_2$ and 21% $O_2$. hMSCs were grown in T175 culture flasks using α-Minimal Essential Medium (α-MEM) supplemented with 16.5% heat-inactivated foetal bovine serum (FBS), 1% L-glutamine and 50μg/mL penicillin-streptomycin (PS) (all from Gibco, Thermofisher) and fed every three days. 20mL of media was used to ensure complete coverage of the T175 flask. hMSCs were grown to 70% confluence before being passaged. hMSCs were lifted from culture by first aspirating the media, washing once with deionised PBS (DPBS) and adding trypsin-EDTA (both from Gibco, Thermofisher) diluted in DPBS to a concentration of 0.05% for 3-4 minutes at 37ºC and 5% $CO_2$. The flasks were then tapped forcefully to encourage detachment. Once detached, an equal volume of $\alpha\text{-MEM}_{16.5\%FBS+PS}$ was added to neutralise the trypsin. The cell solution was then centrifuged at 1200rpm for 5 mins and the supernatant aspirated. The pellet was resuspended in a volume of media equating to 1mL of $\alpha\text{-MEM}_{16.5\%FBS+PS}$ per T175 flask of cells before performing a cell count.

### 2.1.2 Cell counting

With the cell solution ready, a cell count was performed using a Neubauer haemocytometer. 20μL of cell solution was mixed with 20μL of trypan blue and 20μL of this mixture was added to the haemocytometer to determine cell number and viability. Excluding trypan blue stained dead cells, cells were counted on the four corner grids and an average was taken. Only live cells were taken into account in the

seeding for experiments. Once counted, MSCs were then sub-cultured for expansion across more T175 flasks or prepared at a suitable concentration for experiments described later.

### 2.1.3 Resuscitation, expansion and freezing of hMSCs

Cryovials containing 1 million MSCs were taken from liquid nitrogen storage and quickly thawed at 37ºC in a water bath for 2 minutes. The 1mL cell suspension was then added to 10mL of pre-warmed $\alpha$-$MEM_{16.5\%FBS+PS}$. The cells were centrifuged at 1200rpm for 5 mins and the supernatant was decanted off, removing the dimethylsulfoxide (DMSO) (Sigma Aldrich). The pellet was resuspended in 1mL of media before being added to a pre-warmed T175 flask and returned to the incubator. If the hMSCs were for expansion, before 24 hours the hMSCs were lifted using trypsin as described earlier, counted and seeded across T175 flasks at a cell density of 60 cells/$cm^2$. Low density seeding helps to maintain the stem/progenitor cell populations of the hMSCs, whereas excessive and prolonged confluency will trigger differentiation of the hMSCs[391-393]. When the cells reached 70% confluence they were detached, pooled, counted and diluted in media to a concentration of 2 million cells/mL. The cell solution was then diluted 1:1 using freezing medium consisting of $\alpha$-$MEM_{50\%FBS+PS}$ and DMSO at a ratio of 9:1, bringing the cell concentration to 1 million cells/mL. 1mL of the cell solution/freezing solution mixture was then added to each cryovial, placed in a freezing container filled with isopropanol and stored overnight at -80ºC. The next morning the cryovials are transferred to liquid nitrogen for long term storage.

## 2.2 Human monocyte-derived macrophage culture

### 2.2.1 Isolation of human monocytes from buffy coats

Buffy coats from single donors were obtained from the Northern Ireland Blood Transfusion Service (NIBTS); these buffy coats are what remains after blood donations are processed to extract the majority of the red cell fraction. Ethical approval for the use of these buffy coats was acquired through the Queen's University Belfast Research Ethics Committee (Ref: 14/35 Title: Investigating regulation of

inflammation and repair in the Acute Respiratory Distress Syndrome using blood cells extracted from buffy coat). This leaves a white cell enriched sample, which is depleted of platelets and a large proportion of red cells. Took blood from buffy coat (approximately 40-65mL) and diluted in HBSS minus $Ca^{2+}$ and $Mg^{2+}$ (Gibco, Thermofisher) up to a volume of 140mL. Added 15mL of Ficoll Paque Premium (GE Healthcare) to a 50mL falcon tube and then slowly layered the blood mixture on top at a 45$^{o}$ angle, being careful not to mix the two layers, to produce four 50mL falcons. Centrifuged at 480g for 20 minutes at 20ºC with the brakes off. This separates the blood components by density centrifugation and produces a white layer containing lymphocytes and monocytes. Used a Pasteur pipette to extract the white cell layer from the tubes taking care not to remove red cells. Pooled the cells from each tube into one falcon before adding HBSS up to 50mL. Centrifuged at 1200rpm for 5 minutes at 4$^{o}$C. Aspirated the supernatant, taking care not to lose the cell pellet. This is done to remove any residual Ficoll that would have been extracted along with the cells. Repeated the wash step with HBSS two more times. Resuspended the cell pellet in 20mL of Roswell Park Memorial Institute (RPMI) (Gibco, Thermofisher) media supplemented with 1% heat-inactivated FBS and 50µg/mL penicillin-streptomycin and prepared an aliquot with a 10x dilution of cells in media. Added 20uL of this to a haemocytometer and place in the incubator for 5 minutes to allow monocytes to adhere. Performed a cell count using a light microscope and haemocytometer, adherent monocytes will appear cloudy/translucent. Seeded the monocytes at the desired density in $RPMI_{1\%FBS+PS}$ for at least 2 hours to allow adherence of the monocytes. All other contaminating cells, e.g. erythrocytes and lymphocytes, will not adhere. Monocytes were seeded at 300,000 per well in 24-well plates and 1,000,000 per well in 6-well plates.

### 2.2.2 Generation of hMDMs from monocytes

Aspirated the $RPMI_{1\%FBS+PS}$ from the adherent monocytes and washed twice with HBSS to remove contaminating cells. Added $RPMI_{10\%FBS+PS}$ further supplemented with 10ng/mL of GM-CSF (R&D Systems, Biotechne). GM-CSF will differentiate the monocytes into macrophages over the 6 to 7-day incubation.

## 2.3 Co-culture experiments

### 2.3.1 Non-contact co-culture of human MSCs and human MDMs

After 6-7 days of differentiation hMDM co-culture with hMSCs could begin. Before beginning the experiment, hMDMs were serum starved in unsupplemented RPMI for 2 hours. MSCs used in experiments were passage 3-5. hMSCs would be suspended in culture above the hMDMs using 0.4μm porous hanging cell culture transwell inserts (Merck Millipore). These required pre-soaking in media for at least 20 mins prior to adding the cells to facilitate cell adherence and to allow co-culture without contact. For all co-culture experiments, hMSCs were prepared at a 5:1 ratio of hMDMs to hMSCs. In some experiments, normal human lung fibroblasts were used as a cell control for the MSCs, to determine whether the effects observed were specific to hMSCs. Conditions were prepared in $RPMI_{1\%FBS+PS}$ with any additional stimuli at a volume of 500μL for 24-well plates and 2mL for 6-well plates.

### 2.3.2 *In vitro* stimulation experiments

To generate an inflammatory environment, *E. coli* LPS O111:B4 (List Biological Laboratories) was added to the hMDMs and hMSCs at a working concentration of 10ng/mL in $RPMI_{1\%FBS+PS}$. In later experiments, to mimic the microenvironment of the alveolar compartment of patients with ARDS, BALF from ARDS patients was used for stimulation in place of LPS. These BALF samples were of those taken from the HARP study performed by Craig *et al* and published in 2011, where they investigated the use of simvastatin as a treatment for ARDS[394]. BALF samples used for these stimulation experiments were baseline samples taken prior to any intervention. BALF from nine of these patients were pooled to generate a stock and the pooled sample was then diluted to 30% in $RPMI_{1\%FBS+PS}$ before stimulation. When possible healthy volunteer BALF was included to demonstrate whether it is ARDS patient BALF or BALF in general that is responsible for the observed effects. Healthy volunteer samples were taken from the study published by Shyamsundar *et al* in 2014 investigating the effects of KGF in mild, LPS-induced lung injury[395]. The healthy volunteer BALF samples used for these stimulations were from individuals who had received neither LPS nor KGF. In some experiments, as a positive control for M2

macrophage activation, recombinant human IL-4 and IL-13 (R&D Systems, Biotechne) were added to hMDMs at a concentration of 10ng/mL.

### 2.3.3 Lactate dehydrogenase (LDH) assay

For pilot ARDS patient BALF stimulation experiments, cytotoxicity assays were performed on the hMDMs using the Cytotoxicity Detection Kit (LDH) (Life Sciences, Roche). hMDMs were cultured in BALF at concentrations of 100%, 50% and 30% for 24 hours. After the 24 hours, the supernatants were lifted and cell debris was removed by centrifugation at 10,000rpm. 50µL of each sample was added in duplicate to wells of a Maxisorp 96-well ELISA plate (Nunc). Assay mixture was prepared by mixing 250µL of the diaphorase/$NAD^+$ catalyst with 11.25mL of the iodotetrazolium chloride/sodium lactate dye solution. 50µL of the assay mixture was added to each well on top of the samples and mixed on a shaker for 1 minute. The assay mixture is pink and will turn red as the assay develops. The 96-well plate is then incubated at room temperature for 30 mins before being read by a spectrophotometer at 490nm. A number of controls are included for this assay; a background control is 100µL of the assay mixture and is used as a blank which is subtracted from all other values. There is an untreated control of hMDMs cultured in $RPMI_{1\%FBS+PS}$ alone to determine basal LDH release. Then there is the cell death control which consisted of $RPMI_{1\%FBS+PS}$ with 2% Triton-X detergent (Sigma Aldrich) to induce total cell death. Cell death was calculated as a percentage relative to the cell death control. Absorbance readings of the background control were subtracted from all other readings. Then the following calculation was used for the test conditions:

$$\text{Cytotoxicity (\%)} = (\text{experimental value/cell death control value}) \times 100$$

## 2.4 Cytokine analysis

### 2.4.1 Enzyme-linked Immunosorbent Assay (ELISA)

Human TNFα, IL-8, KGF, adiponectin, Ang-1, lipocalin-2 and TIMP3 levels in cell supernatants were quantified using ELISA duoset kits (R&D Systems, Biotechne). Exact working concentrations of the kits constituents may be found in the

manufacturer's instructions. Capture antibody for the cytokine of interest was prepared in 1xPBS (Gibco, Thermofisher) to the desired concentration and 100µL of this solution was added to each well of a Maxisorp 96-well ELISA plate (Nunc). The plate was then sealed and incubated overnight at room temperature. The capture antibody was aspirated and the wells washed using wash buffer (1xPBS with 0.05% Tween-20 (Sigma Aldrich)) three times, blotting against clean paper towels after each wash. Care was taken to remove as much liquid as possible after the final wash when blotting. Non-specific binding was prevented by blocking the plates with 300µL of reagent diluent per well (1xPBS with 1% bovine serum albumin (BSA) (Sigma Aldrich)) for at least 1 hour. Again, the plates were washed three times with wash buffer and blotted before addition of the cytokine standards and supernatant samples. A two-fold dilution series was used to prepare a range of standard concentrations and then 100µL of these and the samples were added to each well for a 2 hour incubation. The wells were washed three times and 100µL of detection antibody prepared in reagent diluent was added for another 2 hour incubation. After another three washes, 100µL of streptavidin-horse radish peroxidase diluted 1:40 in reagent diluent was added to each well, and incubated for 20 mins protected from light. A final three washes was performed and 100µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Life technologies) was added and incubated for 20 mins, or until the standards had suitably developed, protected from light. 50µL of 2M sulphuric acid stop solution was added to each well and the plate gently tapped to ensure mixing. The ELISA was then immediately analysed using the Versamax spectrophotometer set to read at 450nm and 540nm wavelength. A subtraction of the wavelength absorbance readings at 540nm was taken from the readings at 450nm for correction. 4-parameter standard curves were produced using Softmax Pro v2.6 and concentrations of samples were extrapolated. TSG6 ELISA was purchased from Cusabio and was performed according to manufacturer's instructions; this assay followed a very similar protocol.

### 2.4.2 Bioplex

The measurement of multiple other cytokines and chemokines was performed using a custom Luminex Performance Assay Multiplex kit (R&D Systems, Biotechne). The analytes for this assay were the cytokines IL-1β, IL-1ra, IL-10, IL-12, IL-23 and IFNγ

and the chemokines CCL5, CCL17, CCL18 and CCL22. The assay was performed according to manufacturer's instructions. The kit was used along with a Luminex analyser that uses a dual laser flow-based sorting and detection platform. Each analyte's antibody is provided already coated onto coloured microparticles. Standards are provided for the analytes and a 3-fold dilution series is used to generate a range. 50µL of either the standard/microparticle or sample/microparticle mixtures were added to the plate provided. The plate was then incubated at room temperature for 3 hours with gentle agitation on a shaker before washing with 100µL of the wash buffer provided with the kit. Wash buffer was then removed with a vacuum manifold dispenser which siphons the liquid through filters on the base of the plate. This wash step was repeated two more times. 50µL of streptavidin-phycoerythrin was added to each well and incubated for 30 mins protected from light on a shaker. Three more washes were performed and a fourth wash was allowed to sit for two minutes before aspiration. There are lasers present in the apparatus which are specific to each of the colour-coded microparticles which should be conjugated to the antibody-analyte complexes. Another laser will excite any of the streptavidin-phycoerythrin fluorophore which has now bound and is present in levels proportional to the amount of analyte in the samples. The plate is then analysed and sample analyte concentrations were deduced by plotting the standard concentrations against the median fluorescence intensity (MFI) using a five parameter logistic fit.

## 2.5 Flow cytometry

### 2.5.1 Preparation for flow cytometry

Flow cytometry was used to assess the purity of hMDMs after isolation and differentiation, analysis of macrophage surface markers, phagocytosis assays and EV characterisation. After the experimental timepoint was reached inserts and supernatants were removed from the hMDMs and the cells were washed with PBS. 1mL of flow buffer (PBS 5%FBS) was added to the cells and they were gently scraped from the well plates using a cell scraper. The cell solution was then added to 5mL polystyrene flow tubes (Sarstedt, Fisher Scientific) and centrifuged at 350g for 5 mins at 4$^{o}$C to pellet the cells. The tubes were then decanted to remove the flow buffer, leaving 200µL in the tube. The cells were resuspended in 0.5mL of flow buffer and

20µL of human FcR binding inhibitor (eBioscience) was added. The cells were incubated on ice for 20 mins to block nonspecific binding. Following this the antibodies **(see Table 2.5.1.1 and Table 2.5.1.2)** would be added at the necessary dilution and incubated for 30 mins on ice, protected from light. For each experiment a number of controls were included, an unstained control, isotypes for each fluorophore in use and single stain controls for each fluorophore. For experimental groups, all antibodies of interest were added to each tube. After the antibody incubation, 3mL of flow buffer was added to each tube and the cells were centrifuged at the same settings as earlier. The supernatant was aspirated and the wash step repeated. After the second wash, 200µL was left in each flow tube and the samples were run. Samples were processed using the FACSCantoII flow cytometer and FACSDiva software (BD Biosciences). Where necessary, compensation between fluorophores was performed on the FACSDiva software by using single stain controls. Data was then analysed using FlowJo v7 software (Treestar). Assessment of purity of hMDMs demonstrated that >95% of cells expressed myeloid specific CD14 and the remaining cells were CD3 positive lymphocytes.

*Table 2.5.1.1: Antibodies used for assessing purity of hMDMs*

| Cell surface marker | Fluorochrome conjugate | Manufacturer |
|---|---|---|
| Anti-human CD14 | PE-Cyanine7 | eBioscience |
| Anti-human CD3 | FITC | eBioscience |

*Table 2.5.1.1: Antibodies used for macrophage markers*

| Cell surface marker | Fluorochrome conjugate | Manufacturer |
|---|---|---|
| Anti-human CD40 | FITC | eBioscience |
| Anti-human CD54 | PE | eBioscience |
| Anti-human CD163 | APC | eBioscience |
| Anti-human CD206 | PE-Cyanine7 | eBioscience |

### 2.5.2 Flow cytometric phagocytosis assay

To determine the phagocytic capacity of hMDMs with or without co-culture of hMSCs, fluorescently labelled pHrodo® green *E. coli* BioParticles® (Thermofisher)

were used. Briefly, after 24 hours of co-culture, the supernatant is removed and the hMDMs are washed twice with PBS. *E. coli* particles were then suspended in $RPMI_{1\%FBS+PS}$ with 10µL of stock per 1mL of media (provides particles at a 20:1 ratio particles to hMDMs). *E. coli* bioparticles were then added to hMDMs in a total volume of 2mL for 6-well plates. The plates were then centrifuged at 350g for 5 mins at 4ºC to ensure contact between the hMDMs and the particles. hMDMs were incubated at 37ºC and 5%$CO_2$ for 90 minutes, protected from light, to allow phagocytosis to occur. A number of wells are not treated with particles and serve as an unstained control. As recommended by the manufacturer, a number of wells were treated with the particles but incubated on ice as a control. These cells should not be capable of phagocytosis and any signals detected in these wells should be subtracted as background. After the incubation, the supernatant was aspirated, and the cells were washed twice in PBS. A final 1mL of PBS was added to each well and the hMDMs are gently scraped. The cell solution was centrifuged at 350g 4ºC for 5 mins and the supernatant aspirated before adding 200µL of PBS to the tube for resuspension. The samples were then analysed by flow cytometry.

## 2.6 Paracrine factor studies

### 2.6.1 Blockage experiments

In order to test the importance of lipocalin-2 (LCN2), IL-6, PGE2, lipoxin $A_4$ and TGFβ in the hMSC effects, their functions were inhibited within hMSC-CM and added to hMDM with LPS. LCN2, IL-6 and TGFβ were neutralised using antibodies obtained from R&D systems at the doses recommended by the manufacturer. hMSCs were first seeded into 24 well plates at 60,000 cells per well (coinciding with the number of cells used in the 1:5 non-contact co-culture experiments with hMDMs) in fully supplemented α-MEM. The following day when the hMSCs had adhered, they were washed with PBS and the media was replaced with $RPMI_{1\%FBS1+1\%PS}$ to generate culture medium (CM). 24 hours later the CM was taken into eppendorfs and centrifuged at 10,000g to remove detached cells and cell debris. The supernatant was lifted and then treated with the antibodies which were incubated for 30-60 mins before addition to the hMDMs along with LPS.

PGE2 production was inhibited using ibuprofen (Abcam) which was added to hMDMs with LPS with or without hMSC-CM at a concentration of 100μM (recommended by manufacturer). Ibuprofen acts to prevent PGE2 production through the inhibition of COX2 and COX1 enzymes[396]. Lipoxin $A_4$ function was blocked using the selective FPRL1 antagonist WRW4 at 10μM (recommended by manufacturer). This was also added to hMDMs along with LPS with or without hMSC-CM. Lipoxin $A_4$ has previously been attributed to the protective effects of hMSCs in endotoxin-induced lung injury[397]. Blockage of the lipoxin $A_4$ receptor using WRW4 substantially mitigated the protective effects of hMSCs in that model. Ibuprofen and WRW4 inhibitors were added to the hMDMs without pre-incubation. For all of these blockage experiments, the hMDMs were cultured with these conditions for 24 hours before lifting the supernatants for cytokine measurement by ELISA.

### 2.6.2 Lipocalin-2 (LCN2) stimulation of hMDMs

To further assess the function of LCN2, 500ng/mL of recombinant human LCN2 (R&D systems) was added to hMDMs along with LPS to assess their inflammatory response. This dose of recombinant LCN2 was previously described in a study on LCN2 function[398].

## 2.7 Extracellular vesicle studies

### 2.7.1 CD44 blockage experiments

As previously demonstrated by Monsel *et al,* CD44 expression on hMSC-EVs was important for the uptake of EVs by recipient cells, namely human monocytes and alveolar type II epithelial cells[171]. To determine if hMSC-EVs were responsible for hMSC effects on hMDMs, blockage studies were performed. hMSC-CM was generated as described in **Section 2.6.1**. The CM was treated with an antibody for human CD44 (mouse anti-human CD44, BD Biosciences) or isotype control (mouse $IgG_{2B}$, R&D Systems, Biotechne) to a final concentration of 3μg/mL or given no antibody. They were incubated for 30mins at 37°C with gentle mixing to allow the antibody to bind any EVs present in the CM and then added on to serum starved hMDMs with 10ng/mL of LPS as described earlier. Importantly CD44 is also

expressed on macrophages[205], so in order to control for any effects of this antibody on hMDMs directly, additional groups were included where hMDMs were treated with the antibodies without hMSC-CM. After 24 hours of stimulation, supernatants were extracted for cytokine analysis.

### 2.7.2 Generation of EV-free FBS

For EV work, media was prepared with heat-inactivated FBS which was free of all contaminating EVs. FBS was taken in 40mL centrifuge tubes (Hitachi) and ultracentrifuged at 100,000g using the Himac CP100WX ultracentrifuge (Hitachi) for 3 hours at 4°C with a P28S rotor (Hitachi). The supernatant was then taken and used to supplement either αMEM or RPMI for treating the MSCs.

### 2.7.3 Isolation of hMSC-EVs

hMSCs were grown to around 60-70% confluence under standard conditions before having their media replaced with 15mL of EV-depleted $\alpha MEM_{16.5\%FBS+PS}$ (for microvesicle characterisation experiments) or $RPMI_{1\%FBS+PS}$ (for addition to MDMs after preparation). The hMSCs were incubated for 48 hours at 37°C and 5%$CO_2$ and the culture medium was collected. Firstly, the medium was centrifuged at 10,000g at 4°C for 20 mins to remove cells and cell debris. The supernatant was then ultracentrifuged at 100,000g for 2 hours at 4°C and the supernatant was aspirated. The pellet of EVs was resuspended in PBS to fill the 40mL tubes before centrifuging again at the same settings. This acts as a wash to minimise contamination with protein aggregates. The PBS supernatant is then discarded and the EV pellet is finally resuspended for downstream processing.

### 2.7.4 Quantification of hMSC-EVs

In a pilot experiment, hMSC-EVs were isolated as described above and the concentration of RNA and protein was assessed using the Nanodrop 2000 (ThermoFisher). Briefly, hMSC-EVs were washed and resuspended in 100μL of PBS. 1μL of this resuspension was added to the Nanodrop 2000. The RNA concentration

was found to be 0.47ng/μL corresponding to 47ng of RNA from the isolation. The EV isolation had a protein concentration of 1470μg/mL corresponding to 147μg of protein in the isolation. These values are similar to those reported by Monsel *et al* when assessing RNA and protein content of their hMSC-EV isolations[171].

### 2.7.5 Characterisation of hMSC-EVs by flow cytometry

To provide an indication of the size of hMSC-EVs, hMSC-CM was processed by flow cytometry with the addition of aldehyde/sulphate latex beads (ThermoFisher) which have a diameter of 4μm. The latex beads provide a reference point to compare with the EVs in FSC vs SSC plots. hMSCs were grown to confluence and stained with a number of different reagents. Stains used for the characterisation of EVs are listed in **Table 2.6.5.1**. hMSCs were treated with human FcR binding inhibitor for 20 minutes at 4ºC to prevent non-specific binding. They were stained with CellMask™ Orange (cell membrane), propidium iodide (necrosis), Annexin V (apoptosis), anti-human CD44 (or isotype control) and MitoTracker® Deep Red FM for 45 minutes at 4 ºC in the dark.

*Table 2.7.5.1: Stains used for the characterisation of hMSC-EVs*

| Stain | Fluorochrome conjugate | Manufacturer |
|---|---|---|
| CellMask™ Orange | PE | ThermoFisher |
| Propidium iodide | PerCP-Cy5.5 | Sigma Aldrich |
| Annexin V | Pacific Blue | Biolegend |
| Anti-human CD44 | PE-Cy7 | eBioscience |
| MitoTracker® Deep Red FM | APC | ThermoFisher |

After the incubation, hMSCs were washed three times in PBS and EV-free $\alpha MEM_{16.5\%FBS+PS}$ was used to prepare CM. After 24 hours, CM was lifted and processed immediately by flow cytometry (FACSCantoII flow cytometer and FACSDiva software). Unstained hMSC-CM was processed first in order to determine the necessary FSC and SSC voltage settings required to detect the EVs. Single stain controls were then tested, as well as the isotype for anti-CD44, to set gates for these stains and to perform the necessary compensation. The gating strategy was as follows:

EVs were gated for by FSC vs SSC; cell membrane positive events were selected using CellMask™; events which were negative for propidium iodide (necrosis) staining were then gated for; Annexin V staining (apoptosis) was excluded. This final gate was then used to determine the extent of CD44 expression by EVs and the proportion of these EVs which contained mitochondria. To provide some information on the size of these EVs, latex beads with a diameter of 4μm were processed by flow cytometry first separately and then in combination with hMSC-CM. FSC vs SSC plots of the latex beads provided a reference point to compare with the size of the EVs.

## 2.8 Animal studies

C57BL/6 male mice (8 to 10 weeks old; Harland Institute, UK) were used. Animals were maintained in the Biological Services Unit (BSU) at Queen's University Belfast. Experiments were sanctioned and approved by the UK Home Office and Queen's University Belfast Ethical Review Committee.

### 2.8.1 Bronchoalveolar lavage (BAL)

Mice were culled with intraperitoneal (IP) injection of 300μL of xylazine/ketamine mixture. The skin of the upper body and neck was cut open and the trachea was exposed. The connective tissue surrounding it was cut away and a length of string was placed in behind the trachea. A small hole was cut in the anterior wall of the trachea and a severed 21 gauge needle was introduced through the opening. The string was then tied around the trachea anchoring the needle in place and 1mL of PBS was instilled into the lungs. The PBS was flushed in and lifted out 7-10 times to ensure thorough sampling of the bronchoalveolar compartment and to isolate as much AMs as possible.

### 2.8.2 *Ex vivo* culture of mouse AMs

A 20μL aliquot of the BALF samples were mixed with 20μL of trypan blue and a cell count was performed **(see Section 2.1.2)**. Murine AMs were seeded into 6 well plates at 3 million per well in the PBS they were extracted in but then spiked with FBS to

generate $PBS_{1\%FBS}$. The AMs were given 4 hours to adhere before they were gently washed with PBS and then treated with hMSC-EVs suspended in $RPMI_{1\%FBS}$ or media alone. For EV-treated AMs, each well was treated with the EVs generated from 10 million hMSCs over 48 hours (approximately ?) (for EV isolation, see **Section 2.6.3**). AMs were cultured *ex vivo* for 48 hours before they were detached using 1mL of Accutase® cell detachment reagent (Innovative Cell Technologies) incubated for 5 mins at 37ºC. Any cells remaining attached were gently dislodged with a cell scraper. The cell viability was determined using trypan blue staining and another cell count was performed. The cells were then centrifuged at 5000g for 5 mins, the supernatant aspirated and the AMs resuspended in PBS such that 35µL of solution contained $2.5x10^5$ AMs.

### 2.8.3 LPS model of lung injury

Mice were first anaesthetised by isoflurane inhalation for 1-2 minutes individually. Mice were given 0.8µg of LPS per gram of body weight. LPS was administered intranasally (IN) diluted in PBS at a volume of 35µL. Control mice were given PBS alone. Mice were observed until they recovered from the isoflurane. 4 hours after the LPS instillation, mice were anaesthetised once again using 100µL of xylazine/ketamine injected using a 25 gauge needle IP. Mice were then treated with either vehicle control (PBS), *ex vivo*-cultured hMSC-EV-treated AMs or untreated AMs ($2.5x10^5$ AMs per mouse) IN in a volume of 35µL. Mice were observed until recovery and returned to holding. 24 hours after LPS instillation, mice were culled and subjected to BAL **(Section 2.9.2)** to be used for cell counts, protein quantification and cytokine analysis.

### 2.8.4 Total cell counts

BALF samples were first diluted 2-fold in PBS into fresh eppendorfs, 20µL of this was mixed with 20µL of trypan blue and then 10µL of this mixture was added to each side of a Countess® cell counting chamber slide (Invitrogen, Thermofisher). Total cell counts were carried out using the EVE™ automated cell counter (NanoEnTek, VWR).

Two counts were taken for each BALF sample and dilutions were accounted for in the final cell counts expressed as cells/mL.

### 2.8.5 Cytospin preparation, imaging and neutrophil counts

After taking the aliquot for total cell counts, BALF samples were centrifuged at 5000g for 5 mins to remove the cells from suspension. Supernatants were lifted and placed in a fresh eppendorf then stored at -20°C for future processing. The cell pellet was resuspended in 300µL of PBS and centrifuged at 10,000rpm for 5 mins using the StatSpin Cytofuge®2 (Beckman Coulter, VWR) onto microscope slides. Slides were given 2 hours to dry and then stained using the Speedy Diff kit (Clin-tech). Slides were given another hour to dry before imaging using the Leica Epifluorescence DM5500 microscope at 20x magnification. Enough images were taken of each slide in order to count a total of 400 cells. Cells were counted using ImageJ software and the percentage of neutrophils was determined. Using this percentage, total neutrophil counts were calculated from the total cell counts taken earlier.

## 2.9 Transfection with anti-miRNA inhibitors

hMSCs were seeded in 12 well plates at 100,000 cells per well and allowed to adhere for 24 hours. The cells were washed with OptiMEM reduced serum media (Invitrogen, Thermofisher) and then another 400µL of OptiMEM was added to the cells before returning them to the incubator. Prepared a tube containing 89µL of OptiMEM and 1µL of anti-miR125b inhibitor or negative control inhibitor per well (yielding a 10nM solution when given to the cells) (Ambion, Thermofisher), mixed and incubated at room temperature for 5 mins. Next prepared a tube with 7µL of OptiMEM and 3µL of oligofectamine transfection reagent (Invitrogen, Thermofisher) per reaction, mixed and added 10µL of this per reaction to the tubes containing the inhibitors. This final mixture was incubated for a further ten minutes before adding 100µL to each well, in a drop-wise fashion, to the hMSCs, totalling 500µL per well. The cells were returned once again to the incubator for 6 hours, at which point an additional 500µL of OptiMEM, this time supplemented with 20% FBS was added to the cells giving a 10%

FBS solution. The cells were kept in culture for 72 hours to allow efficient transfection.

## 2.10 RNA processing and real-time polymerase chain reaction (RT-PCR)

### 2.10.1 RNA isolation

hMSCs were seeded at 100,000 cells per well in 12 well plates and processed for transfection as described in **Section 2.7.1**. To show the efficiency of the transfection, total RNA was isolated, miRNA was reverse transcribed to cDNA and RT-PCR was performed. RNA isolation was carried out using the RNeasy mini kit (Qiagen) as per manufacturer's instructions. After the transfection, hMSCs were washed in PBS and then 350µL of RLT lysis buffer supplemented with β-mercaptoethanol (inhibits the action of RNase enzymes, 10µL per 1mL of lysis buffer) was added to each well. To ensure complete lysis of cells, scraped the base of each well using a fresh cell scraper and then collected the lysates in a sterile eppendorf. Samples were homogenised by passing the solutions through a 25 gauge microneedle 10 times. Further processing of RNA was maintained at 4$^{o}$C throughout. An equal volume of 70% ethanol was added to the lysates and the solutions were mixed before placing in the spin columns and centrifuging at full speed for 30 seconds. The flow through was discarded and 700µL of RW1 buffer was added. Columns were centrifuged again and the flow through was aspirated. Next 500µL of RPE buffer, supplemented with ethanol, was added and again centrifuged. This RPE buffer wash was repeated one more time and then the collection tube holding the spin column replaced with a fresh one before centrifuging once again. This step is used to ensure that all ethanol has been removed from the columns, as ethanol interferes with the final RNA elution step. The spin columns are then placed in a final collection eppendorf and 30µL of RNase free water is placed in the column. A final centrifugation extracts a pure, concentrated RNA sample.

### 2.10.2 Quantification of RNA

Total RNA purity and concentration was determined using the Nanodrop 2000. The software was first initialised and then a baseline blank was established by placing 1µL of RNase free water on the platform of the device. After setting a blank, 1µL of the RNA samples were then added and analysed. Between sample additions, the platform was cleaned using a paper towel soaked with 70% ethanol to prevent any sample mixing. Two readings were taken for each sample and an average was calculated. A 260/280 purity ratio of 1.8 or higher was considered pure enough for downstream processing. Before proceeding to reverse transcription, the concentrations of all samples were first normalised to the lowest sample concentration of that batch using RNase free water.

### 2.10.3 Reverse transcription of miRNA

Reverse transcription was carried out using the TaqMan advanced miRNA cDNA synthesis kit (Thermofisher) following the manufacturer's instructions. The first step is polyadenylation; 2µL of the RNA sample was mixed with 3µL of a cocktail consisting of buffer, ATP and the polyadenylation enzyme. The samples were then heated to 37$^{o}$C for 45 mins and 65$^{o}$C for 10 mins for the reaction. Next is ligation, where a mixture of buffer, ligation adaptor, RNA ligase and polyethylene glycol was added to the sample. This reaction was performed at 16$^{o}$C for 1 hour. Reverse transcription was achieved by adding buffer, a mixture of deoxynucleotides, universal RT primer and RT enzymes to the samples and heating to 42$^{o}$C for 15 mins followed by 5 mins at 85$^{o}$C. The last step for this kit was amplification of the newly synthesised miRNA cDNA. This step is important for detection of lowly expressed target miRNAs by RT-PCR and importantly this step does not discriminate between different miRNAs and so amplifies all of them equally preserving their relative expression in the samples. A cDNA master mix and primer mix was added to the cDNA and heated to 95$^{o}$C for 5 mins to activate the polymerase enzyme. cDNA was denatured at 95$^{o}$C for 3 seconds then annealed and extended at 60$^{o}$C for 30 seconds (repeated for 14 cycles). Amplification was stopped by heating to 99$^{o}$C for 10 minutes. cDNA was then used immediately for RT-PCR or kept for long term storage at -80$^{o}$C.

### 2.10.4 RT-PCR

RT-PCR was carried out using TaqMan universal PCR master mix (mRNA) or universal PCR master mix, no AMPErase UNG (miRNA) along with TaqMan primer assay (mRNA) or TaqMan advanced miRNA assay primers (Thermofisher). PCR reactions were prepared in a 96 well plate with a total volume of 20µL per reaction:

| Reagent | Volume (per reaction) |
|---|---|
| Master mix (2X) | 10µL |
| Primer assay (20X) | 1µL |
| cDNA diluted in $ddH_2O$ | 9µL |

For every PCR plate prepared, a no template control and a no reverse transcription control was included. The no template control contained master mix, the primers of interest and only $ddH_2O$. The no reverse transcription control contained master mix, primers and an equal amount of RNA which had not been converted to cDNA. Each sample was added in duplicate for each assay being run. Once the plate had been loaded, the wells were sealed with strip caps, the plate was mixed on a shaker for 30 seconds and then centrifuged at 1500rpm for 2 mins to collect the solutions to the base of each well. The plate was loaded into a Stratagene Mx3005P PCR device (Agilent Technologies) and the RT-PCR reaction was setup like so:

| Step number | Condition settings | Number of cycles |
|---|---|---|
| 1 | 50°C for 2 mins | 1 |
| 2 | 95°C for 10 mins | 1 |
| 3 | 95°C for 15 secs<br>60°C for 1 min | 50 |

## 2.11 Mitochondrial studies

### 2.11.1 Studying mitochondrial transfer

hMSCs were grown to confluence and pre-stained with MitoTracker® Red for 45 mins at 37ºC before being washed three times with PBS. hMDMs were treated with hMSC-CM (prepared for 24 hours after staining) or cultured with hMSCs on Transwell inserts

for 24 hours. hMDMs were washed with PBS before being taken up from culture and prepared for flow cytometry. hMDMs were assessed for positivity of MitoTracker® Red staining, indicative of uptake of hMSC mitochondria. To provide further evidence of mitochondrial transfer, fluorescence microscopy was used. hMSCs were once again pre-stained with MitoTracker® Red and washed with PBS. CM was prepared from pre-stained hMSCs for 24 hours. hMDMs had their nuclei pre-stained with Hoechst 33342 (Sigma Aldrich) and their mitochondria pre-stained with MitoTracker® Green (Thermofisher) for 45 mins at 37ºC. hMDMs were also washed with PBS before treating with hMSC-CM and incubating the hMDMs for 24 hours to allow EV uptake. hMDMs were washed once more with PBS and treated with fully supplemented RPMI. Fluorescence imaging of live hMDMs was performed using the EVOS® FL Auto Imaging System (Life technologies) at 20x magnification.

### 2.11.2 Generating dysfunctional mitochondria

Loss-of-function experiments were performed to assess the importance of mitochondrial transfer from hMSCs in their effects on hMDMs. In order to produce dysfunctional mitochondria, hMSCs were treated with rhodamine 6G (Sigma Aldrich) at a concentration of 1μg/mL for 48 hours at 37ºC as previously described[399]. Because mitochondrial respiration would be blocked by this treatment, hMSCs had their media supplemented to support glycolysis. Standard $\alpha$-$MEM_{16.5\%FBS+PS}$ was supplemented further with uridine to produce a concentration of 50μg/mL and sodium pyruvate to a concentration of 2.5mM. After 48 hours, hMSCs were washed three times with PBS before generating CM for experiment. To further study the importance of mitochondrial respiration in hMDM function, hMDMs were treated with 3μg/mL of oligomycin which was prepared in DMSO and added to $RPMI_{1\%FBS+PS}$. DMSO alone was used as a vehicle control.

### 2.11.3 Assessing mitochondrial respiration

To determine the efficiency of rhodamine 6G as a mitochondrial inhibitor and to assess the effect of hMSC-CM on hMDM mitochondrial activity, the Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies) was used. hMSCs or hMDMs were seeded into

XF 96-well tissue culture plates at densities of 10,000 and 30,000 cells per well respectively and allowed to adhere overnight. The cells were then treated with the various conditions for 24-48 hours depending on the experiment. During this incubation a cartridge plate provided with the kit, which contained the probes for measuring oxygen consumption, was soaked in XF calibrant solution and left to incubate at 37ºC and 0% $CO_2$. Cells were washed twice with XF basal medium which had been supplemented with 10mM glucose, 1mM pyruvate and 2mM glutamine. 180µL of XF basal medium was added to each well and the cells were placed in the incubator at 37ºC and 0% $CO_2$ for 1 hour. Meanwhile the oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and rotenone/antimycin-A inhibitors were prepared as per the manufacturer's instructions. The probe cartridge was collected from the incubator and the inhibitors were added to specific ports of these cartridges. 20µL of oligomycin was added to port A, 22µL of FCCP was added to port B and 25µL of rotenone/antimycin-A was added to port C for each well. These inhibitors would be sequentially injected into the cell solution during the Mito Stress Test. The loaded cartridge plate was then placed in the XF$^e$96 Extracellular Flux Analyser (Agilent Technologies) for calibration. After calibration, the XF 96-well tissue culture plate containing the cells was added to the machine and the Mito Stress Test was performed. Oxygen consumption rate (OCR) readouts produced by the assay were analysed using the Wave software (Version 2.2) (Agilent Technologies).

## 2.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software. Experiments for each hMDM donor were performed in triplicate; the average of these three technical replicates was taken as a single donor replicate. These average values were taken as a single data point for each donor and pooled together for statistical analysis. Pooled data was presented as the mean with standard deviation. Normality was determined using histograms of frequency distribution. For parametric data, Student's t-test (two groups) or one way ANOVA (three or more groups) with post-hoc analysis using Bonferroni's selected comparisons was performed. For non-parametric data, Kruskal-Wallis with post-hoc analysis using Dunn's selected comparisons was used. Statistical significance was regarded as $p<0.05$.

# Chapter 3

# hMSCs promote an unconventional M2-like phenotype in hMDMs in *in vitro* models of ARDS

## 3.1 Introduction

hMSCs represent a promising candidate as a cell-based therapy for ARDS. Their mechanisms of effect in the context of lung injury have been a topic of great interest in the past decade. Previously, MSCs have been shown to act through the production of numerous paracrine factors including soluble mediators, microvesicles and contact-dependent mechanisms[94, 101, 116, 137, 171, 317, 318, 397, 400]. Whilst the effectors produced by MSCs have been well studied, their cellular targets and the precise influence it has on them has not been so thoroughly investigated. The AM is the key immune cell of the lung and their polarisation has been shown to play an important role in the pathophysiology of lung injury with a predominance of the pro-inflammatory M1 macrophage during the acute or injurious phase and an increased proportion of the M2 macrophage during resolution[273-275, 285]. This would suggest that the AM represents a good therapeutic target given that promotion of the M2 anti-inflammatory macrophage phenotype may reduce the extent of inflammation and injury in ARDS. When characterising macrophage phenotypes, one important element to consider is their secretory profile. M1 macrophages will produce pro-inflammatory cytokines and chemokines including TNFα, IL-12, CCL5 and CXCL10 whereas M2 macrophages will secrete anti-inflammatory factors such as IL-10, CCL17 and CCL22 in order to facilitate their divergent functions[212, 213, 220, 266]. Variations in cell surface marker expression also provide information regarding macrophage phenotype. Examples include CD163, which is found in some M2 macrophage phenotypes or CD40 in M1 macrophages[213, 401].

It is important to appreciate the complexity of macrophage polarisation; macrophages are often described as inhabiting a M1 or M2 phenotype but these are extremes and represent collective groups containing many unique subtypes of macrophage. It is therefore unsurprising that certain macrophage phenotypes may exhibit elements of both M1 and M2 classes such as the tumour associated macrophage described by Tsai *et al* in a mouse cancer model. This macrophage had high levels of iNOS and Arginase-1 expression, markers typical of M1 and M2 macrophages respectively[402]. MSCs ability to influence immune cells including macrophages is well established. MSCs have been shown to promote a more regulatory M2-like macrophage phenotype

in models such as asthma and sepsis[287, 403, 404]. The effect of MSCs on AMs in the context of ARDS has not been so well studied. Another important functional feature of macrophages is their phagocytic capacity; macrophage polarisation can significantly influence phagocytosis. Macrophage phagocytosis represents an important functional aspect of their phenotype and has significant implications in ARDS of infectious aetiology. These include sepsis and pneumonia-induced ARDS which are the most common and devastating cases[86, 405]. The ability of macrophages to phagocytose apoptotic neutrophils is diminished by LPS stimulation and this was dependent on the resulting TNFα production by the macrophage[406]. However LPS-treated peritoneal macrophages are more efficient in clearance of opsonised erythrocytes, suggesting that M1 macrophages excel at the phagocytosis of red blood cells[407]. Human MDMs were shown to bind and phagocytose virulent *Mycobacterium tuberculosis (M.tuberculosis)* strains through the use of complement receptors and the mannose receptor (CD206)[408]. Conversely, a predominant M2 macrophage phenotype present in chronic rhinosinusitis with nasal polyps was associated with poorer clearance of *Staphylococcus aureus (S.aureus)*[409]. It is clear then that phagocytosis is influenced by macrophage activation state. Many of the earliest studies investigating macrophage polarisation were performed in mice. While informative, the relevance to human macrophage biology is limited; certainly both innate and adaptive immunity of mice and man differ to some degree[410]. There is an increasing effort to switch to primary human macrophage studies *in vitro*[213, 411]; even *in vivo* mouse models are being 'humanised' in a bid to increase the relevance of our research by introducing human cells into mice[412, 413]. This is essential in drawing more meaningful conclusions from our research which are more translatable to human disease.

## 3.2 Aims

The main objective for this chapter was to study the effect of hMSC non-contact co-culture on hMDM phenotype in *in vitro* models of ARDS. The inflammatory stimuli chosen to simulate those present in ARDS were *E. coli* LPS and the BALF from patients of ARDS. The main objective of this chapter can be divided into three specific aims:

- To investigate the effect of hMSCs on the hMDM cytokine and chemokine profile
- To investigate how hMSCs modulate expression of surface markers associated with M1 and M2 macrophage phenotypes
- To determine the effect of hMSCs on hMDM phagocytosis

## 3.3 hMSCs reduce TNFα and IL-8 secretion by hMDMs stimulated with LPS without cell contact

hMDMs were stimulated with LPS for 24 hours with or without the co-culture of hMSCs or fibroblasts. Fibroblasts were included as a cell control to determine whether MSC effects are cell specific. Fibroblasts were chosen as they are also of mesenchymal lineage but lack the stem/progenitor population found in MSCs responsible for their multipotency. IL-4/13 were included as a positive control for M2 macrophage activation. The supernatants were extracted and the levels of TNFα and IL-8 were analysed by ELISA. TNFα and IL-8 are key pro-inflammatory cytokines associated with ARDS pathophysiology and severity[20, 27, 414]. The LPS produced a robust response from the hMDMs and both cytokines were secreted. Results show that the levels of TNFα are reduced by MSCs (2465.1±1102.6 vs 1038.1±206.7 pg/mL) ($p<0.05$) and fibroblasts (2465.1±1102 vs 1157.5±236.8 pg/mL) ($p<0.05$) in non-contact co-culture suggesting an anti-inflammatory effect on the hMDMs by both cell types **(Fig 3.1 A)**. IL-8 levels were decreased by hMSCs (11180.7±2957.7 vs 8107.2±2355.3 pg/mL) and fibroblasts (11180.7±2957.7 vs 9670.7±3984.2 pg/mL) but this did not reach statistical significance **(Fig 3.1 B)**. Importantly, when the same number of hMSCs used in co-culture are cultured alone and stimulated with LPS they also produce IL-8 (6172±86.9 pg/mL, n=2, data not shown).

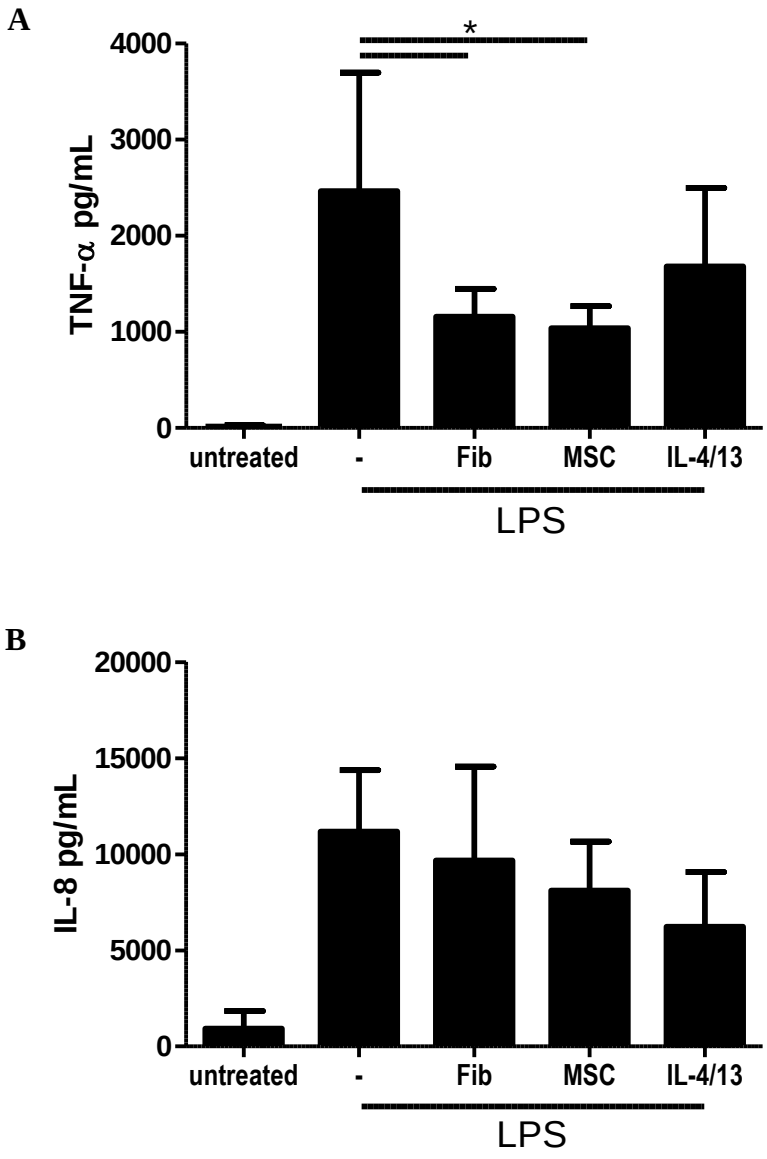



**Fig 3.1 hMSCs reduce TNFα secretion by hMDMs stimulated with LPS without cell contact**

Supernatants from LPS-treatd hMDMs were extracted and the levels of TNFα and IL-8 were determined by ELISA. Error bars show standard deviation (SD). (A- n=3-5 per group, B- n=3-7 per group) (One way ANOVA with Bonferroni's post hoc test, *$p<0.05$).

### 3.4 hMSCs suppress both M1 and M2-associated cytokine/chemokine secretion by hMDMs in the presence of LPS

To further characterise the MSC-induced macrophage phenotype, supernatants produced from the LPS non-contact co-culture experiments described in **Section 3.3** were also analysed by bioplex array. This array included the M1 macrophage-associated cytokines and chemokines IL-1β, IL-12, IL-23, IFNγ and CCL5 as well as the M2 macrophage-associated cytokines and chemokines IL-1ra, IL-10, IL-17, CCL17, CCL18 and CCL22[211, 266]. hMSC co-culture resulted in reduced levels of CCL18 (904.3±604.3 vs 292.2±190.3 pg/mL) ($p<0.05$) and CCL22 (106210.6±67672.8 vs 22486.0±13266.5 pg/mL) ($p<0.05$) **(Fig 3.2 E and F)**, had no effect on the amount of IL-1ra, IL-23, CCL5 and CCL17 produced **(Fig 3.2 A-D)** and IL-1β, IL-10, IL-17 and IFNγ were below detection limits (data not shown). This data demonstrates that hMSCs also induce a downregulation of both M1 and M2 macrophage-associated chemokines produced by hMDMs in the presence of LPS. The positive control for M2 activation IL-4/IL-13 strongly upregulated CCL17 and CCL18 production in LPS-treated hMDMs **(Fig 3.2 D and E)**. No statistical analysis could be performed on these groups however because only two donors were treated with the IL-4/IL-13 condition.

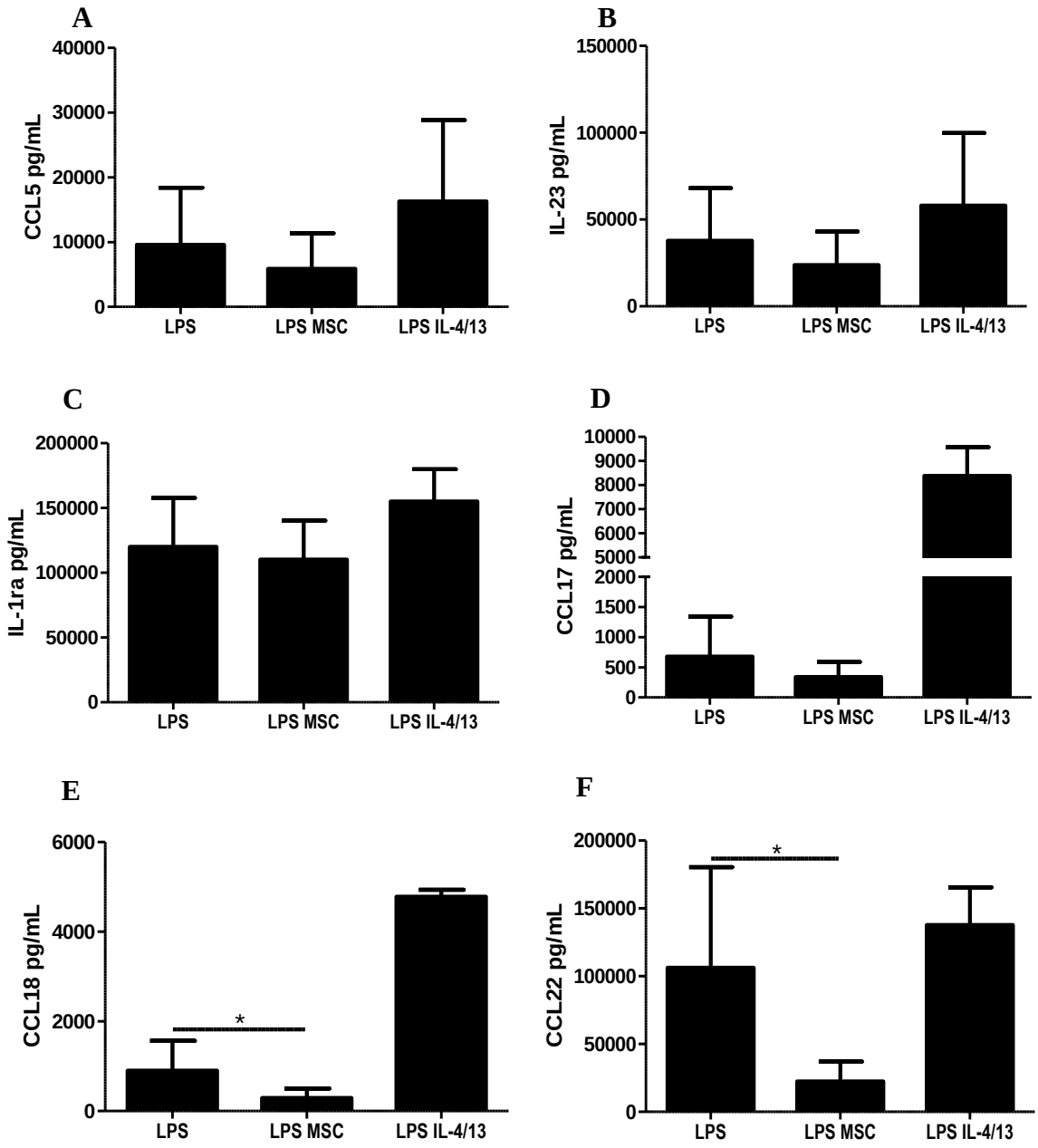



**Fig 3.2 hMSCs suppress both M1 and M2-associated cytokine/chemokine secretion by hMDMs in the presence of LPS**

Supernatants from LPS-treated hMDMs were extracted and tested by bioplex for levels of IL-1ra, CCL17, CCL18, CCL22, (M2) IL-23 and CCL5 (M1). Error bars represent SD (n=6 for LPS and LPS MSC groups, n=2 for LPS IL-4/13 group) (One way ANOVA with Bonferroni's post hoc test, except CCL17 which was analysed by Kruskal-Wallis with Dunn's post hoc test, *$p<0.05$).

### 3.5 hMSCs induce expression of the M2 macrophage marker CD206 in hMDMs in the presence of LPS

Next cell surface marker expression profile of hMDMs in co-culture with hMSCs was investigated. Initially, hMDMs were assessed for their expression of well recognised M2 macrophage polarisation markers CD200R1, MHC class II and transglutaminase 2[260, 415, 416] as well as M1 markers CD64 and CD80[417, 418] by RT-PCR. Preliminary data from these experiments showed that hMSCs had no effect on the expression of any of these markers by hMDMs (data not shown). hMDMs were then stained for established M1 markers CD40 and CD54 as well as the M2 markers CD163 and CD206[213, 401, 419, 420] and analysed by flow cytometry. The total percentage of cells positive for each of these markers was quantified and the MFI was determined. The expression of the M1 markers CD40 and CD54 was not affected by the presence of MSCs in co-culture by either percentage of cells positive for expression or MFI **(Fig 3.3 A and B)**. The M2 marker CD163 expression levels were very low in LPS treated hMDMs and this was not altered by the presence of hMSCs **(Fig 3.3 C)**. However, CD206 marker expression was upregulated by hMSCs. hMSCs increased the proportion of hMDMs expressing CD206 by an average of 7-fold (23.3±9.5 vs 63.5±16.3 %) ($p<0.05$) and whilst the MFI of its expression was increased by 10-fold (361.5±97.8 vs 1672.3±1119.6), this did not reach statistical significance **(Fig 3.3 D)**. Notably, CD206 was the only marker in this panel that was influenced by IL-4/IL-13 treatment, the positive control for M2 macrophage activation, suggesting that it may be the only reliable indicator of M2-like macrophage polarisation in this *in vitro* system. Of course IL-4/IL-13 are associated with M2a-type polarisation specifically[220] and other M2-inducing stimuli may have influenced these additional markers.

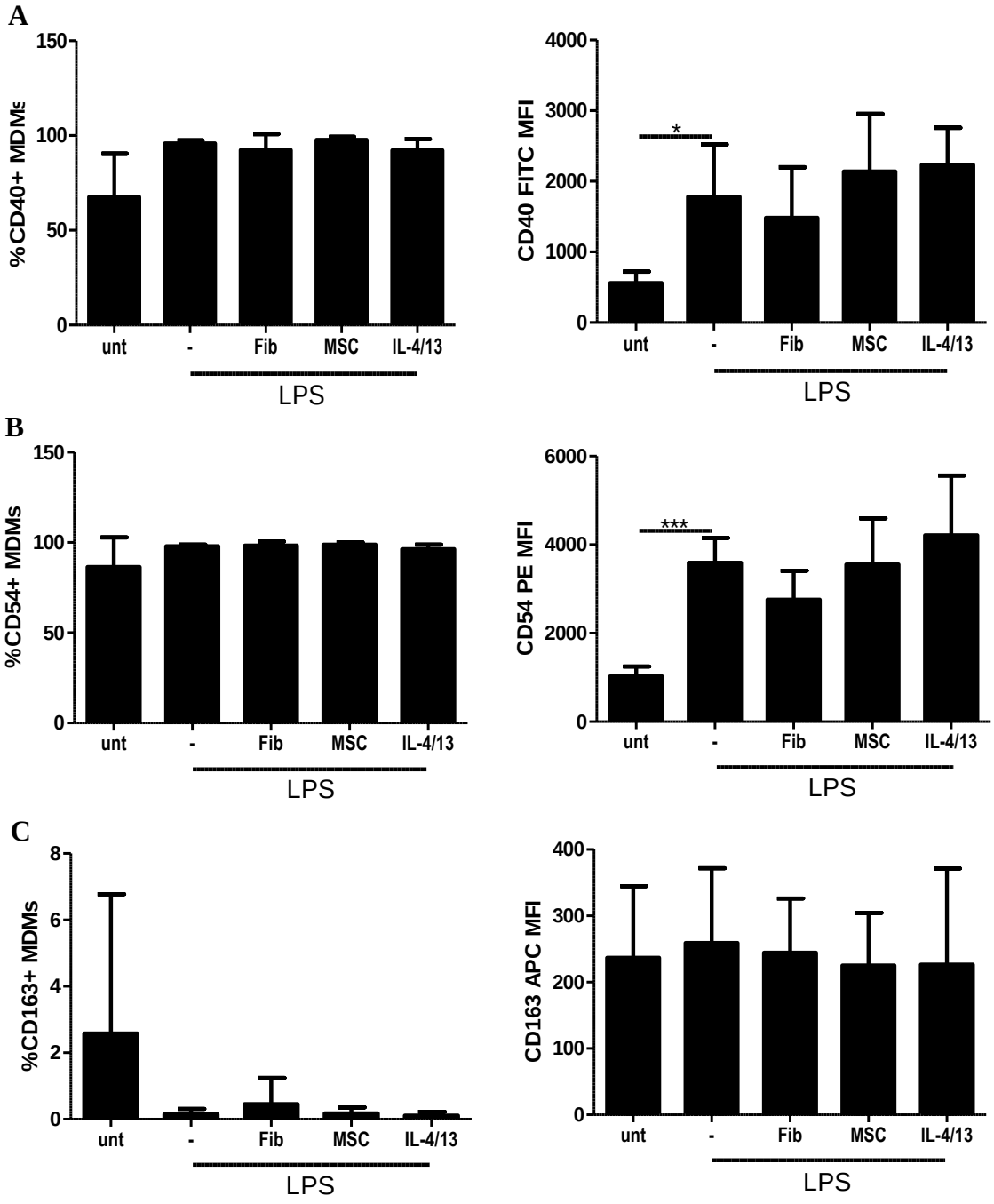



**Fig 3.3 hMSCs induce expression of the M2 macrophage marker CD206 in hMDMs the presence of LPS (continued overleaf)**

After stimulation with LPS for 72 hours hMDMs were stained for M1 markers CD40 (A) and CD54 (B) and M2 markers CD163 (C) and CD206 (D, overleaf) and analysed by flow cytometry. Data is presented as both percentage positive cells and the MFI for each marker. Error bars represent SD (n=4 for all groups, except for LPS and IL-4/13 group which is n=3) (One way ANOVA with Bonferroni's post hoc test, *$p<0.05$ ***$p<0.001$).

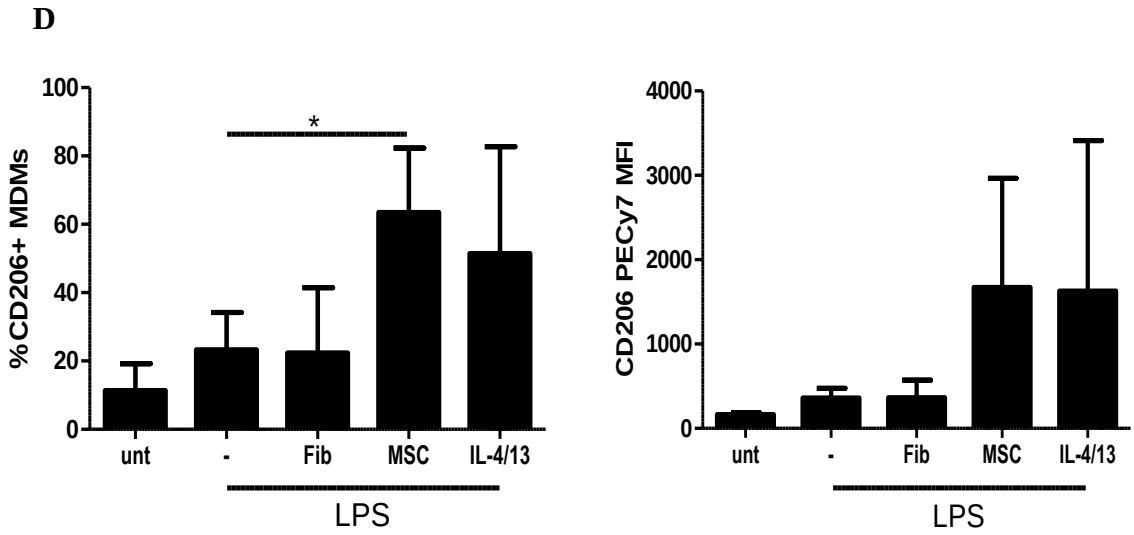



**Fig 3.3 (continued) hMSCs induce expression of the M2 macrophage marker CD206 in hMDMs the presence of LPS**

Data is presented as both percentage positive cells and the MFI for each marker. Error bars represent SD (n=4 for all groups, except for LPS and IL-4/13 group which is n=3) (One way ANOVA with Bonferroni's post hoc test, *$p<0.05$).

### 3.6 hMSCs increase the proportion of phagocytic hMDMs stimulated with LPS

hMDM function with LPS stimulation was further studied using a flow cytometric *E. coli* bioparticle phagocytosis assay. The total percentage of hMDMs which ingested *E. coli* bioparticles and the extent of this phagocytosis was determined by flow cytometry. LPS induced a non-significant decrease in the percentage of phagocytic hMDMs (12.3±5.0 vs 7.2±3.4 %). hMSCs were then able to increase the proportion of phagocytic hMDMs in the presence of LPS (7.2±3.4 vs 21.2±4.9 %) ($p<0.01$) **(Fig 3.4 A)**. Phagocytic MFI, determined by FITC fluorescence, was unaffected by LPS and had a small trend towards increasing in the hMSC group **(Fig 3.4 B)**.

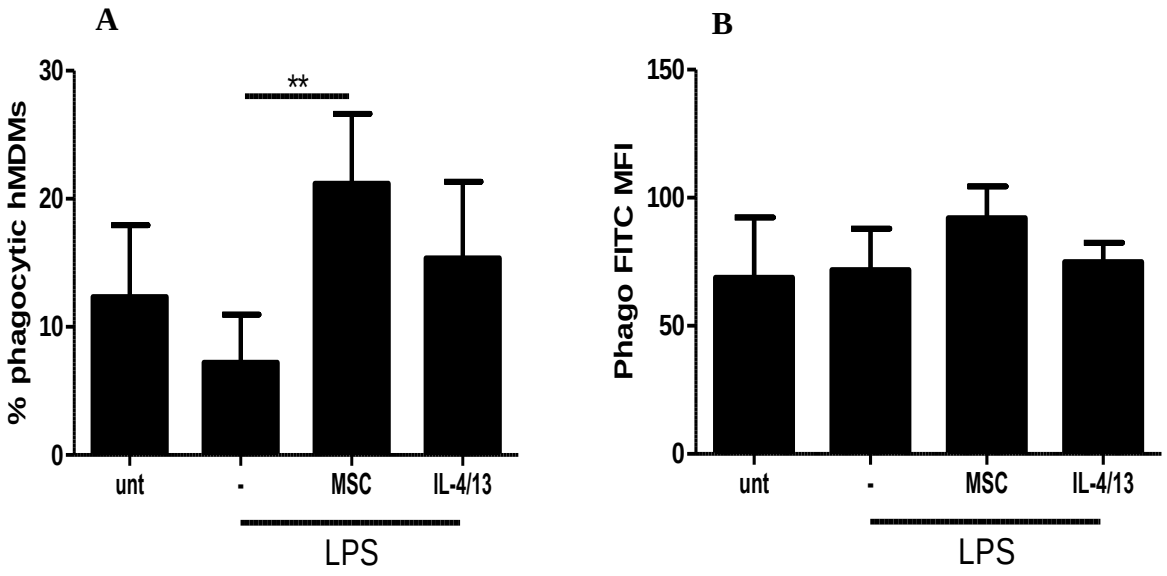



**Fig 3.4 hMSCs increase the proportion of phagocytic hMDMs stimulated with LPS**

hMDM phagocytic activity was assessed using *E. coli* bioparticles and flow cytometry. Data is presented as percentage of macrophages which have phagocytosed bioparticles (A) and MFI (B) as a measure of the extent of phagocytosis. Error bars represent SD (n=5 for all groups except IL-4/13, which is n=4) (One way ANOVA with Bonferroni's post hoc test, **$p<0.01$).

### 3.7 hMSCs reduce TNFα production by hMDMs stimulated with BALF from patients with ARDS

Before beginning cytokine analysis, I first performed a dose response using the pooled BALF samples. hMDMs were treated with 100%, 50% and 30% BALF for 24 hours and cytotoxicity was determined by LDH assay. A large degree of cell death occurred in the 100% and 50% concentrations but 30% BALF did not induce cell death of the hMDMs (data not shown). hMDMs were treated with pooled BALF from patients with ARDS diluted to 30% volume in $RPMI_{1\%FBS+PS.}$ They were stimulated for 24 hours in the presence or absence of hMSCs in co-culture at a 1:5 ratio of hMSC to hMDM. Once again, hMSCs were placed on porous transwell inserts hanging above the hMDMs. After 24 hours the supernatants were taken and TNFα levels were quantified by ELISA as before. The pooled BALF itself was also assayed to determine the levels of TNFα that could be attributed to the samples and therefore was not produced by the hMDMs in culture. There was no detectable TNFα present in both the healthy volunteer BALF and the ARDS BALF (data not shown). In some pilot experiments healthy volunteer BALF was included as a control to show that the stimulation is specific to ARDS patient samples. Healthy volunteer BALF elicited a small TNFα response from the hMDMs (97.3±3.9 pg/mL). Pooled BALF from patients with ARDS elicited a more pronounced but still very mild TNFα response (230.2±30.3 pg/mL). The presence of hMSCs in non-contact co-culture reduced hMDM TNF-α production to levels comparable with the healthy volunteer BALF-treated group (109.1±37.4 pg/mL) **(Fig 3.5)**.

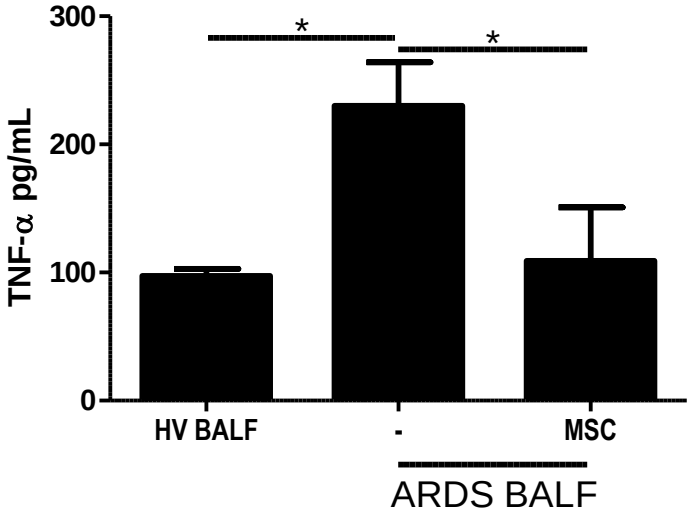



**Figure 3.5 hMSCs reduce TNFα production by hMDMs stimulated with BALF from patients with ARDS**

Supernatants were collected and the levels of TNFα were quantified by ELISA. HV-healthy volunteer. Error bars represent SD (healthy volunteer BALF n=3, ARDS BALF and ARDS BALF + hMSC groups n=5) (Kruskal-Wallis with Dunn's post-hoc test, *$p<0.05$).

### 3.8 hMSCs increase expression of the M2 marker CD206 by hMDMs in the presence of ARDS patient BALF

hMDMs were treated with pooled BALF for 72 hours. After stimulation the supernatants were aspirated and the cells washed before preparing them for flow cytometric analysis of CD206 expression. hMDMs cultured in $RPMI_{1\%FBS+PS}$ alone exhibited similar CD206 expression as those treated with ARDS BALF. hMSC co-culture resulted in an increase in the percentage of hMDMs expressing CD206 with ARDS BALF stimulation (24.6±3.3 vs 56.0±8.1 %) ($p<0.01$) **(Fig 3.6 A)**. The MFI of CD206 expression was also increased by hMSCs (254.4±62.1 vs 629.8±129.3) ($p<0.01$) **(Fig 3.6 B)**.

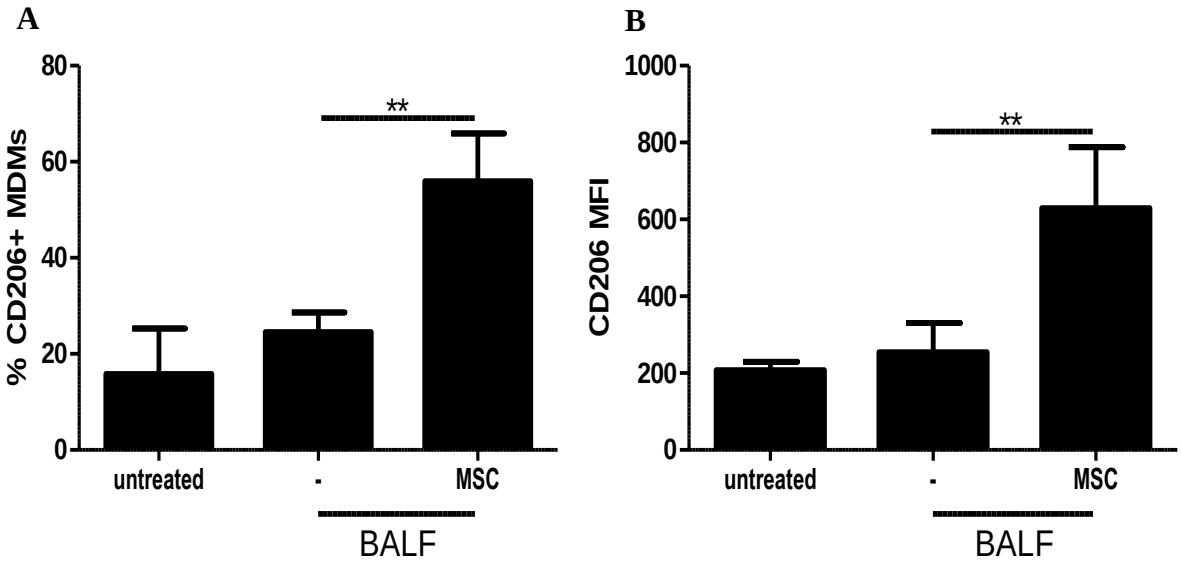



**Fig 3.6 hMSCs increase expression of the M2 marker CD206 by hMDMs in the presence of ARDS patient BALF**

hMDMs were analysed by flow cytometry for the expression of the CD206 surface marker. Expression is presented as the percentage of hMDMs which are positive for CD206 (A) and the MFI of CD206 expression (B). Error bars represent SD (n=3 all groups) (One way ANOVA with Bonferroni's post hoc test, **$p<0.01$).

### 3.9 hMSCs enhance the phagocytic capacity of hMDMs stimulated with ARDS patient BALF

hMDMs were treated with ARDS BALF for 24 hours with or without hMSC in co-culture. After 24 hours the supernatant was aspirated, the cells were washed and treated with *E. coli* bioparticles for the analysis of phagocytic capacity by flow cytometry. ARDS BALF had no effect on the percentage of phagocytic hMDMs. hMSCs were able to increase this percentage (14.4±2.1 vs 27.0±3.7 %) ($p<0.01$) **(Fig 3.7 A)** but not the MFI **(Fig 3.7 B)**.

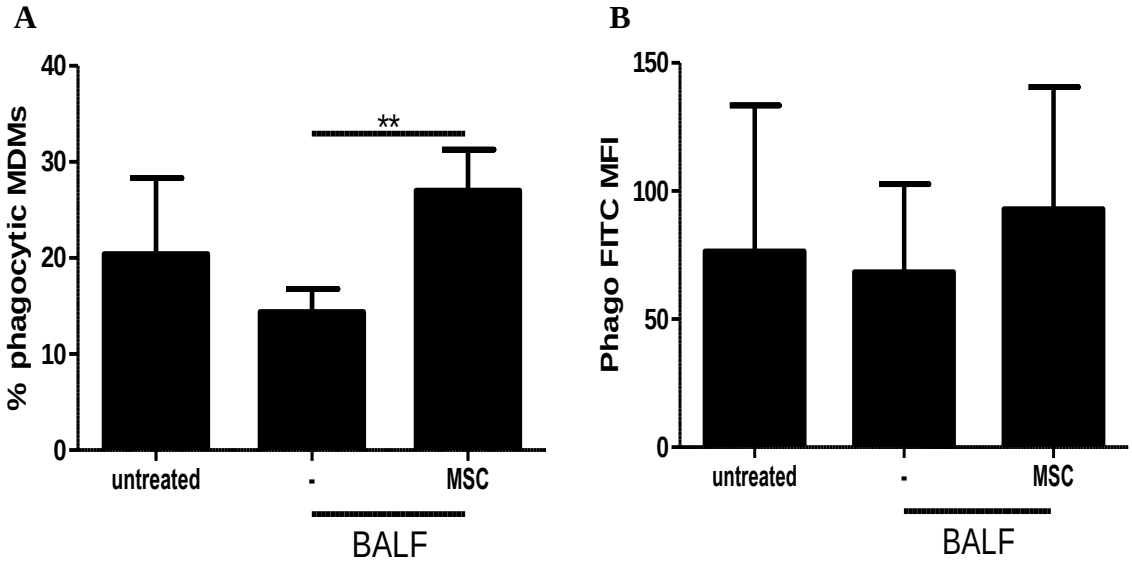



**Fig 3.7 hMSCs enhance the phagocytic capacity of hMDMs stimulated with ARDS patient BALF**

hMDMs were treated with ARDS BALF for 24 hours with or without hMSCs. hMDMs were treated with *E. coli* bioparticles and phagocytosis was quantified by flow cytometry. Phagocytic capacity is expressed as the percentage of hMDMs which had phagocytosed bioparticles (A) and the MFI representing the phagocytic index (B). Error bars represent SD (n=4 all groups) (One way ANOVA with Bonferroni's post hoc test, **$p<0.01$).

### 3.8 Discussion

MSC regulation of macrophage phenotype and function has been studied previously; the general consensus is that they promote a regulatory phenotype with increased phagocytic capacity; my results are mostly consistent with these reports[286, 287]. This data demonstrates that hMSCs are capable of influencing hMDM phenotype in the presence of the inflammatory stimulus LPS. Firstly, hMSCs exhibited the ability to downregulate a number of cytokines and chemokines produced by hMDMs. These results demonstrate a substantial decrease in the amount of TNFα produced by hMDMs when hMSCs are in co-culture without contact. Total IL-8 levels were also decreased by hMSCs albeit not significantly, however it is important to remember that the hMSCs themselves produced IL-8 and so it is still possible that they were effectively reducing IL-8 production by the hMDMs. This data taken alone would suggest an anti-inflammatory effect of the hMSCs on the hMDMs. TNFα and IL-8 are two key pro-inflammatory cytokines associated with ARDS pathophysiology[414, 421, 422]. It is possible then that reducing levels of such cytokines may reduce the extent of inflammation and injury in these patients leading to improved outcomes. Of course this is an *in vitro* model with only hMSCs and hMDMs present; *in vivo* there are also lung parenchymal cells and microvascular cells to consider. Pulmonary microvascular endothelial cells isolated from patients of ARDS are known to produce higher levels of IL-8 than those of control individuals[423]. Furthermore, AM-derived TNFα induces secretion of many pro-inflammatory mediators from alveolar type II pneumocytes including MCP-1, IL-6 and keratinocyte chemoattractant (KC), the murine equivalent of IL-8[424]. These cells all contribute to the inflammatory milieu in the alveolar compartment and the effects of MSCs on inflammatory functions of these cells are also of importance. A number of studies have been published demonstrating the ability of MSCs to reduce levels of pro-inflammatory cytokines in preclinical models of sepsis and lung injury[73, 76, 141]. However, these preclinical studies do not identify the cells being influenced by MSCs in order to reduce cytokine levels. The findings in this chapter corroborate the findings observed by Kim and Hematti in hMDMs where MSC co-culture was found to reduce intracellular TNFα levels[287]. Notably, the normal human lung fibroblasts were also able to reduce TNFα production by hMDMs. It is perhaps not surprising that the fibroblasts were capable of exerting a similar anti-inflammatory effect, as fibroblast immunomodulation has been reported in the past.

Fibroblasts, when stimulated with T lymphocyte-derived IFNγ, produce indoleamine 2,3-dioxygenase (IDO) and actively suppress T cell proliferation[425]. This suggests that immunomodulation may be a trait common to cells of mesenchymal lineage but these are *in vitro* based studies and so we cannot assume that fibroblasts would be efficacious *in vivo.* Indeed, while MSC therapeutic effects in preclinical models *in vivo* or *ex vivo* are widely reported, fibroblasts given alongside them have not been able to create the same effects[76, 82].

The bioplex data shows a largely unchanged secretory profile with downregulation of two M2-associated chemokines, CCL18 and CCL22. While the secretion of IL-23, CCL5 and CCL17 were also decreased, this was not statistically significant. IFNγ, IL-1β, IL-10, IL-12 and IL-17 levels were below the limits of detection of the assay for hMDMs in all conditions. Whilst not all cytokines and chemokines investigated in this chapter have been implicated in ARDS pathophysiology, they have all been associated with macrophage polarisation towards either the M1 or M2 macrophage phenotypes. CCL18, also known as dendritic cell-specific CC chemokine and alternative macrophage activation-associated CC chemokine 1, is a chemoattractant for naïve T cells and B cells[426, 427]. CCL22, or macrophage-derived chemokine, is able to recruit regulatory T cells, NK cells, monocytes and dendritic cells[428, 429]. CCL18 and CCL22 have not been implicated in ARDS but they do appear to play a role in pulmonary fibrosis. CCL18 is produced by AMs in patients of idiopathic pulmonary fibrosis (IPF) which acts to accelerate collagen synthesis by human lung fibroblasts and CCL22 is another prevalent chemokine in patients of IPF which is associated with poorer outcomes[430, 431]. Given that fibrosis is an element of late stage ARDS that hinders the proper regenerative process of the alveolus, it could be speculated that decreased levels of CCL18 and CCL22 may benefit patients of ARDS.

My hypothesis was that hMSCs would induce an M2-like human macrophage phenotype. While pro-inflammatory cytokine production was decreased, there was no upregulation of any M2-associated anti-inflammatory cytokines or chemokines. On that basis it seems unjustified to refer to this macrophage phenotype as anti-inflammatory if there is no evidence that the macrophages themselves can exert anti-

inflammatory effects. Rather it seems that the hMSCs have promoted a suppressed macrophage phenotype with a dampened secretory profile reducing the pro-inflammatory capacity of the macrophages. To the best of my knowledge, there has been no macrophage phenotype previously described which shares all of these traits but some comparisons can be made to other studies. A tolerant M2-like macrophage phenotype can be promoted by repeated exposure to TLR ligands such as LPS, defined by a lack of TNFα response to subsequent treatments and no IL-10 response either[432]. The comparison could also be made between the MSC-induced phenotype shown here and that of unchallenged macrophages. It was unexpected that the levels of IFNγ, IL-1β, IL-10, IL-12 and IL-17 would be so low given the body of literature reporting production of these cytokines by macrophages[433-436]. However the lack of such responses in these hMDMs could perhaps be explained by the stimulus used. This was an *in vitro* model of sterile inflammation with only *E. coli* LPS present. This TLR4 ligand alone may not be sufficient to induce all of these M1 macrophage-associated cytokines. For example, there is evidence that human monocytes may produce IL-1β with TLR4 stimulation alone however macrophages require a concomitant ATP stimulation for proper inflammasome activation and processing of pro-IL-1β[434]. IFNγ and IL-12 are primarily associated with intracellular infections by pathogens including *Mycobacterium tuberculosis* and more potent IL-12 induction occurs after priming with IFNγ[433, 437]. IL-10 production is also very low in these cultures which may be a reflection of the monocyte differentiation protocol used here (culture in GM-CSF for 6 to 7 days). Minimal IL-10 production by human macrophages has been reported in GM-CSF-differentiated, LPS-stimulated cells whereas levels are higher in M-CSF cultured MDMs with the same LPS treatment[438].

Next the effect that hMSCs have on hMDM surface marker expression in the presence of LPS was investigated. No effect was observed on the expression of established M2 markers MHC class II, CD200R1 and transglutaminase 2[260, 415, 416] or M1 markers CD64 and CD80 by hMSCs determined by RT-PCR. The marker panel was extended to investigate M1 markers CD40 and CD54 as well as M2 markers CD163 and CD206 by flow cytometry to further characterise the hMSC-induced hMDM phenotype. hMSCs were able to increase expression of the M2 marker CD206 but had no effect

on CD163 expression or either of the M1 markers CD40 and CD54. The absence of modulation of many macrophage markers by the various conditions in this study may highlight the lack of robust, validated markers for the characterisation of human macrophage phenotype. The hMSC-induced increase in expression of CD206 has previously been attributed to IL-6 production by the MSCs in systemic lupus erythematosus (SLE)[439]. CD40 ligand expressed on activated T cells binds with CD40 on macrophages to elicit IL-12 secretion, another M1 macrophage function[440]. Based on these functions, CD40 has been used as a marker of M1 human macrophages in a number of studies[213, 411]. CD54, or intercellular adhesion molecule (ICAM-1), belongs to the immunoglobulin supergene family and mediates cell adhesion to other cells as well as matrix components. CD54 contributes to leukocyte extravasation through interactions with its ligand LFA-1 and serves as a co-stimulatory molecule in the activation of Th1 cells[419, 441, 442]. ICAM-1 is constitutively expressed but its expression level is increased by pro-inflammatory cytokines including TNFα, IL-1β and IFN-γ owing to NF-κB transcriptional regulation[443-445].

As mentioned earlier, CD163 is a haemoglobin/haptoglobin scavenger receptor capable of eliciting an IL-10 response by monocytes and has been established as a marker of alternatively activated human macrophages[265, 411, 446]. The mannose receptor, CD206, is amongst the most commonly used M2 macrophage markers[411, 418]. Ambarus *et al* demonstrated that CD163 is selectively upregulated in human macrophages using IL-10 as the polarising stimuli and that IL-4 potently induces CD206 expression[418]. In this study, MSCs increased expression of CD206 but not CD163 in human MDMs and consistent with Ambarus' findings using IL-4, my positive control of IL-4 and IL-13 together also induced CD206 expression but not CD163. Notably, IL-10 was not detected by bioplex in co-culture experiments which is consistent with the low CD163 expression. Very few of the MDMs in my experiments expressed CD163 regardless of the condition which may be a result of the GM-CSF based differentiation protocol used, which has been reported before[447]. In the same report, van der Does *et al* describe reduced CD163 expression in M-CSF differentiated macrophages in response to LL-37, an anti-microbial peptide produced by MSCs[96]. It is perhaps unsurprising then that CD163 expression was low in these MDMs and that MSCs did not act to enhance its expression. Jaguin and colleagues

conducted a thorough study characterising human macrophage polarisation using a variety of different stimuli[448]. Their results show that CD206 expression did not distinguish between M1 and M2 macrophages polarised using IFN-γ/LPS and IL-4 respectively. The present study does not show a significant increase in CD206 expression by MFI either, although this may have been achieved with data from more donors. The disparity in these results could be explained by the culture methods for isolated monocytes; Jaguin *et al* had used M-CSF as opposed to GM-CSF. This could have resulted in the difference in the percentage of CD206 expressing macrophages at baseline. Jaguin *et al* reported 63.5% of unstimulated macrophages expressing CD206 whereas an average of less than 20% was observed in this study. The M1 markers CD40 and CD54 were expressed on virtually all MDMs that were stimulated with LPS and to a higher degree than unstimulated cells. Neither MSCs nor IL-4 and IL-13 had any effect on this expression either by percentage or MFI. It is known however that macrophages may exhibit both M1 and M2 macrophage properties. For example, adipose tissue macrophages of mice given a high fat diet adopt an M1/M2 phenotype characterised by enhanced IL-12p40 and IL-1β production yet diminished iNOS and CD86 expression (M1), along with increased Arg-1 and IL-1ra levels (M2)[449]. Importantly, CD206 was the only marker analysed which was influenced by either hMSCs or the IL-4/IL-13 positive control suggesting that this may be the only reliable marker for alternative macrophage activation in this *in vitro* system. Inclusion of additional M2-inducers including IL-10 or glucocorticoids may have influenced these markers. Also of note, while fibroblasts matched the TNFα suppressing capacity of hMSCs, they were not able to upregulate CD206 expression by hMDMs suggesting that hMSC and fibroblasts do not exert identical effects on macrophage phenotype.

Next the effect that hMSCs had on hMDM phagocytosis was investigated through the use of *E. coli* bioparticles. LPS treatment resulted in a slight but non-significant decrease in the proportion of phagocytic MDMs whereas hMSCs were able to increase this. It is difficult to compare these results to the literature given the diversity in the macrophage source, culture methods, stimulation and the phagocytic target that are published. These results complement the work of Michlewska *et al* showing that LPS and subsequent TNFα production inhibit macrophage phagocytosis although this was of apoptotic neutrophils and not *E. coli*[406]. LPS was shown to increase clearance of

opsonised red blood cells by peritoneal macrophages[407]. It is generally accepted that M1 pro-inflammatory macrophages are more adept in their antimicrobial functions compared to M2 macrophages. But this is not to suggest that M2-like macrophages are inefficient in phagocytosis or bereft of antimicrobial function in general. Smythies *et al* describe human intestinal macrophages exhibiting "inflammatory anergy" evidenced by their lack of IL-1, IL-6, IL-10, TGFβ and TNFα production, yet they showed substantial phagocytosis and killing of *Salmonella typhimurium (S. typhimurium)* and *E. coli*[450]. hMSCs have been repeatedly shown to enhance bacterial clearance in a number of preclinical disease models of sepsis and lung injury[77, 83, 84, 451]. These effects could be partially attributed to production of antimicrobial factors including LL-37 and lipocalin-2 but the ability of MSCs to enhance the phagocytosis of blood monocytes and alveolar macrophages is also vital[77, 83, 84, 96].

The LPS experiments suggest that hMSCs are able to promote an M2-like hMDM phenotype with enhanced phagocytic activity. LPS is a pathogen-associated molecular pattern (PAMP) that likely contributes to inflammation in patients with ARDS triggered by the likes of *E. coli* and *P. aeruginosa*, previously reported to account for 14% and 17% of bacterial infections respectively[452]. Of course LPS alone does not replicate live bacterial infection. Furthermore, AMs *in vivo* would also be exposed to cytokines and other such factors produced by alveolar epithelial cells and invading neutrophils which would accumulate in the alveoli. In order to more closely mimic the complex microenvironment that exists in the alveolar space in ARDS, hMDMs were treated with ARDS patient BALF. BALF from these patients has been shown to contain factors including TNFα, IL-8, matrix metalloproteinase (MMP)-2, MMP-9 and elastase[414, 453, 454], providing a more representative stimulus to the hMDMs. Chastre *et al* reported that BALF from ARDS patients with ventilator-associated pneumonia can also test positive for cultures of *S. aureus* and *P. aeruginosa*[455]. It is important to remember though that due to the nature of the BAL procedure, the BALF will present these various factors to the hMDMs at a lower concentration. This is not the first study to use human samples for *in vitro* experiments. In 1969, Robinson *et al* collected urine samples from 50 individuals, either healthy or with various types of leukemia, to test their effects on the growth of bone marrow colonies[456]. Human saliva was used in *in vitro* wound closure assays to determine the factors responsible

for the accelerated wound closure observed in the oral cavity compared to the skin[457]. Human BALF samples have also been used experimentally. To study the effects of antenatal steroids and chorioamnionitis on alveolar epithelial wound repair in the context of preterm infants, BALF was used in an *in vitro* scratch assay[458]. Another group pooled the BALF taken from five patients with cystic fibrosis and used it to stimulate peripheral blood neutrophils to elucidate the effects on TLR2, 4 and 5 expression[459]. The ARDS BALF samples used in the present study were taken from the HARP study published by Craig *et al* and the healthy volunteer BALF samples were taken from the simvastatin study carried out by Shyamsundar *et al*[394, 395]. These samples are described in **Section 2.3.2** of the Materials and Methods.

hMDMs stimulated with ARDS BALF produced modest levels of TNFα. The extent of this TNFα response was substantially lower than that of LPS-treated hMDMs. Notably, the concentration of LPS used (10ng/mL) was very high and so a pronounced inflammatory response would be expected. In the case of the ARDS BALF, the low TNFα production could be explained by the dilute nature of these samples. In the HARP study from which these BALF samples were taken, the BAL was performed by instilling three separate 50mL aliquots of saline into a portion of the right middle lobe[394]. 150mL is a large volume and this dilution could explain the mild inflammatory response seen in this experiment. Furthermore, the BALF was diluted in media to 30% by volume to prevent cytotoxicity. There are a number of potential factors contained in the ARDS patient BALF samples which could have been responsible for activating the hMDMs. This could have been anything from inflammatory cytokines to bacterial products. TNFα was not deteactable in the BALF samples alone. This is incongruous with Suter *et al's* finding showing that during severe ARDS, patients could present with levels of TNFα averaging approximately 10ng/mL. In the same study they observed vast quantities of soluble TNF receptors I and II (sTNFR) in patient BALF[414]. Furthermore, mild ARDS severity averaged 376pg/mL of TNFα in the BALF. Park *et al* found that TNFα was only detectable in the BALF of 28.6% of patients with established ARDS at day 1, a value which decreased with time[460]. The use of 30% BALF, the course of freeze/thaw cycles that the samples have undergone over time and the possibility of sequestration of TNFα by its endogenous decoy receptors, sTNFRI and II, may explain the apparent absence of

this cytokine in the BALF. The absence of TNFα however does not necessarily suggest that there were no pro- or even anti-inflammatory cytokines present that may have influenced the hMDM response. For example the endogenous IL-1β antagonist, IL-1ra, is abundant in ARDS patient BALF[454]. IL-10 is also detectable in ARDS BALF and lower levels of these anti-inflammatory mediators was associated with increased mortality[461].

In healthy volunteer BALF-treated hMDMs there was also TNFα produced (97.3pg/mL). BALF, regardless of the source, will likely have some form of innocuous material present. The AM is inherently tolerant to these substances and have a relatively high threshold for activation, an advantageous quality which prevents excessive and unnecessary inflammation in the airways. This threshold is maintained by the endogenous levels of TGFβ produced in response to their interactions with alveolar epithelial cells[196]. These hMDMs *in vitro* would not encounter this inhibitory TGFβ signal which could have resulted in the small TNFα response seen here. Importantly, the presence of hMSCs in co-culture resulted in the reduction of TNFα production by hMDMs treated with ARDS BALF. This mirrors the findings of the LPS experiments and suggests that hMSCs are able to reduce pro-inflammatory cytokine secretion by human macrophages when exposed to the inflammatory milieu of the distal lung in ARDS. Flow cytometry revealed that hMSCs were able to increase CD206 expression in hMDMs stimulated with ARDS BALF based on both total percentage positivity and the extent of expression (MFI). When we compare the effects of LPS and BALF on hMDM CD206 expression as well as the effects of hMSCs in both cases, the two results are quite comparable. Neither LPS nor ARDS BALF had any effect on CD206 expression compared to untreated control hMDMs. Additionally, hMSCs increased the percentage of CD206 positive hMDMs from approximately 20% to 60% in each case. The only considerable difference between the LPS and BALF experiments is the change in MFI of CD206 expression with hMSC co-culture. hMSCs increased CD206 MFI non-significantly in LPS but significantly in BALF due to the extent of donor variation. However the average CD206 MFI in LPS-treated hMDMs was much higher than BALF-treated hMDMs. This is likely a result of the variation in stimuli. One explanation could be proposed from the apparent difference in the potency of each stimulus. ARDS BALF induced a much smaller TNFα response than

LPS, and it may be that the hMSCs modulatory functions were not activated to the same degree in BALF as they were in LPS. MSCs are known to be very responsive to signals from their environment. For example, MSCs that are primed with sphingosine-1-phosphate have increased efficacy compared to non-primed MSCs in an animal model of pulmonary artery hypertension[462]. A study by Herold *et al* demonstrated a progressive increase in the proportion of CD206-expressing M2 macrophages with time in pneumonia-associated ARDS patients[463]. This complements an animal study describing the dynamics of macrophage populations in the lung during lung injury, depicting an increased prevalence of M2 macrophages during the later resolution stage[285]. The BALF samples used in the current study were taken immediately after recruitment (within 48 hours of diagnosis) which would be during the acute or injurious phase where M1 macrophages predominate[273]. These results suggest that hMSCs promote an M2-like phenotype in hMDMs which were exposed to the pro-inflammatory stimuli within ARDS BALF. Shifting AMs towards the M2 phenotype at an earlier stage may reduce the severity of lung injury and improve outcomes in ARDS.

Lastly, hMSCs were able to increase the proportion of phagocytic hMDMs in the presence of ARDS BALF. As was observed with LPS, the BALF alone resulted in a trend towards downregulation of phagocytic activity, which was reversed and then enhanced by hMSCs. It has previously been shown that clearance of pulmonary *P. aeruginosa* in septic mice is greatly hindered compared to non-septic mice. AMs isolated from septic mice were less able to ingest and kill *P. aeruginosa ex vivo* and this phenomenon was partly due to elevated IL-10 in the lungs of these mice[464]. As mentioned earlier, ARDS patient BALF may contain IL-10 which could explain the trend towards decreased phagocytosis seen in these experiments. The hMSCs ability to enhance hMDM phagocytosis supports similar findings studying phagocytosis by human monocytes *in vitro* and human AMs *ex vivo*[83, 171]. It is promising that hMSCs are able to enhance hMDM phagocytosis in the presence of ARDS BALF. This may be of great importance in sepsis and pneumonia-induced ARDS. There are limitations to the experiments described in this chapter. *E. coli* LPS is a potent inflammatory stimulus but LPS alone does not replicate the complexity of the alveolar inflammatory milieu in ARDS. The use of ARDS BALF much more closely resembles a clinically

relevant inflammatory stimulus for these hMDMs. However for the present study 30% BALF was used instead of 100%; a dilution of an already dilute sampling of the bronchoalveolar compartment of the patient. The clinical relevance of this *in vitro* treatment is limited by this fact although it is still considerably more relevant than other *in vitro* approaches to model ARDS. The strength of these BALF studies is that they incorporate a plethora of soluble mediators that occur *in vivo*. On that note, given the complexity of these patient samples, it cannot be deduced what specific elements are actively stimulating the hMDMs and hMSCs; this makes it difficult to perform mechanistic studies with the aim of identifying hMSC-derived factors responsible for the shift in macrophage phenotype. For that reason, as well as the scarce and valuable nature of these BALF samples, subsequent experiments will return to the use of LPS as an inflammatory stimulus to elucidate the MSC mechanism(s).

### 3.9 Summary and conclusions

The conclusions from this chapter are:

- hMSCs suppress pro-inflammatory cytokine secretion and M2-associated chemokine secretion in the presence of LPS
- hMSCs increase the expression of the M2 macrophage surface marker CD206 with LPS
- hMSCs enhance the proportion of phagocytic hMDMs with LPS treatment
- hMSCs can modulate TNFα secretion, increase CD206 expression and enhance the phagocytosis of hMDMs in the presence of ARDS patient BALF

Taken together, these results suggest that hMSCs are capable of promoting an unconventional M2-like macrophage phenotype in hMDMs in *in vitro* models of ARDS. This hMSC-induced M2-like macrophage phenotype is characterised by a dampened secretory profile, upregulated CD206 expression and enhanced phagocytic capacity. Promotion of this phenotype in AMs could represent one mechanism by which hMSCs exert their beneficial effects in lung injury, mediating increased bacterial clearance and reduced inflammation.

# Chapter 4

# Investigating the mechanism(s) by which hMSCs modulate hMDM phenotype and function

## 4.1 Introduction

The previous chapter characterised the macrophage phenotype that is induced by hMSCs without contact in the presence of *E. coli* LPS and BALF from patients with ARDS. The next aim of this project was to elucidate the mechanism(s) by which they exert these effects. Due to the diverse functions of hMSCs including differentiation, regeneration and immunoregulation[56, 465, 466], there is great interest in their potential therapeutic applications. Certainly, they have been tested in a number of clinical trials ranging from acute GVHD to Crohn's disease to ARDS[122, 123, 131]. In order to facilitate such a broad range of therapeutic effects in several unique inflammatory and degenerative diseases, the modes of action for MSCs must be complex and multifactorial. Another important element of MSC biology is their responsiveness to environmental stimuli, allowing them to tailor their responses to a particular setting. The list of immunomodulatory factors produced by MSCs is extensive. In response to IFNγ, MSCs produce IDO which will inhibit T-cell proliferation[57]. Galectin-1 also contributes to the suppression in T-cell responses and reduced TNFα, IFNγ and IL-2 production by peripheral blood mononuclear cells[467]. PGE2 was found to mediate the switch towards an anti-inflammatory, IL-10-producing macrophage phenotype in sepsis[85]. MSCs also communicate with peritoneal macrophages through TSG6, which binds to their CD44 receptor, diminishing TLR-2-dependent NF-κB activation[468]. As expected, MSCs can exert more pronounced effects when in contact, evidenced by more potent T-cell inhibition; this could be a result of contact-mechanisms in addition to soluble mediators[469]. Contact may also facilitate the transfer of mitochondria from MSCs to neighbouring cells enhancing their bioenergetics[116, 117, 318].

MSC engraftment rates are very low in models of lung injury[91, 470] and this suggests that paracrine factor secretion is the primary mode of action for MSCs *in vivo*. For that reason, this project is focusing on the non-contact-dependent modulation of hMDM phenotype by hMSCs. Determining which factors are responsible for the effects seen on hMDMs is difficult because MSCs produce such a large variety of soluble mediators. A number of potential candidates were tested before determining that mitochondria-containing EVs were facilitating many of these effects **(Chapter 5)**. The

current chapter describes the negative results that were obtained when testing several candidates. The factors initially considered were IL-6, TGFβ, IL-10, PGE2, lipoxin $A_4$, adiponectin, Ang-1, lipocalin-2 (LCN2), TIMP3 and TSG6. IL-6 has previously been shown to play a role in MSC beneficial effects in lung injury and is capable of exerting anti-inflammatory functions[105, 107]. MSCs also produce TGFβ[471], a cytokine capable of decreasing macrophage nitric oxide and TNFα production[472]. While IL-10 is known to be a potent inducer of M2 macrophages[473] and mediates MSC immunomodulatory effects[474], IL-10 was not detectable by bioplex in **Chapter 3** and was not investigated further. Nemeth *et al's* seminal paper in the field demonstrates that MSC-derived PGE2 induces anti-inflammatory IL-10 producing macrophages in an animal model of sepsis[85]. Lipoxin $A_4$ production by MSCs was found to help facilitate MSC protective effects in endotoxin-induced lung injury[397]. Lipoxin $A_4$ is capable of inhibiting NF-κB to reduce TNFα expression by macrophages[475]. Adiponectin and lipocalin-2 are often referred to as adipokines; they are produced by MSCs and have both been shown to modulate macrophage function[77, 476]. Adiponectin is known to induce a more M2-like macrophage profile defined by increased CD206 and IL-10 expression along with decreased TNFα production and attenuated ROS production[477]. A previous study by our group highlights the critical importance of LCN2 secretion by murine MSCs in facilitating their anti-microbial effects in *E. coli*-induced pneumonia *in vivo*[84]. LCN2 also promotes an M2-like profile through activation of PPARγ and is capable of mitigating LPS-induced pro-inflammatory cytokine secretion[398]. Ang-1 is best known for its roles in the maintenance of blood vessel homeostasis through reducing permeability and providing survival signals[478, 479]. Its capacity for promoting barrier integrity also extends to the alveolar epithelium; MSCs overexpressing Ang-1 are more effective in preventing permeability and protein leakage into the BALF than unmodified MSCs in LPS-induced lung injury[141]. Interestingly, Ang-1 appears to have anti-inflammatory functions; it has been shown to block NF-κB activation in LPS-treated macrophages by signalling through the Tie2 receptor and can block IL-8 production by endothelial cells[480, 481]. TIMP3 deletion was shown to promote TNFα production by increasing the activity of TNFα-converting enzyme[482]. TIMP3 is also produced by MSCs and represented another potential candidate for the suppression of TNFα by MSCs[483]. TSG6 has previously been accredited with a number of MSC anti-inflammatory functions including decreased NF-κB activation in macrophages[97, 468]. *In vivo*, TSG6

exerts its anti-inflammatory activity by forming complexes with inter-alpha-inhibitor, a regulator of plasmin which propagates inflammation partly through inducing M1 polarisation in monocytes[484, 485]. TSG6 knockdown in hMSCs prevented their anti-inflammatory effects almost entirely in LPS-induced lung injury[99]. With such a diverse secretome, consisting of cytokines, chemokines, adipokines, anti-microbial peptides and EVs, deciphering the mechanisms of MSC effect is a challenging task.

## 4.2 Aims

The second aim of this project was to determine the mechanism of the hMSC effect on hMDMs. A literature search identified a number of potential candidate factors produced by MSCs which are known to modulate macrophage function. Candidates were chosen which showed evidence of producing an M2-like macrophage phenotype with reduced pro-inflammatory cytokine secretion, resembling the phenotype that was have observed with hMSC co-culture.

### 4.3 IL-6 and TGFβ

hMDMs were treated with LPS for 24 hours with non-contact co-culture of hMSCs. Anti-IL-6 or anti-TGFβ antibody were added to co-culture wells to determine the effect of IL-6 and TGFβ on LPS-induced TNFα production by hMDMs. LPS triggered a TNFα response in hMDMs which was reduced by hMSCs in non-contact co-culture (1093.8 vs 823.9 pg/mL). Anti-IL-6 antibody had no effect on the suppression of TNFα production by hMSCs (823.9 vs 849.3 pg/mL) and neither did anti-TGFβ antibody (823.9 vs 835.6 pg/mL) **(Fig 4.1)**. This result suggested that neither IL-6 nor TGFβ are playing a role in hMSC modulation of hMDM responses in this *in vitro* system.

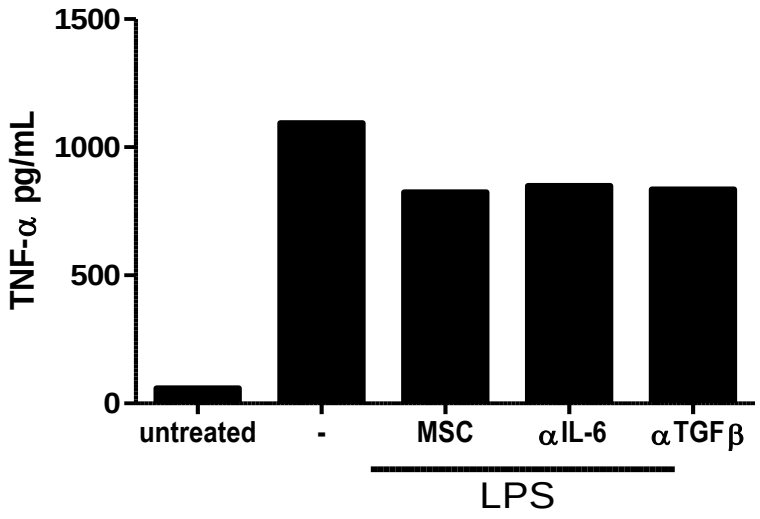



**Figure 4.1 IL-6 and TGFβ**

Supernatants were taken from LPS-stimulated hMDMs after co-culture with hMSCs in the presence or absence of anti-IL-6 or anti-TGFβ neutralising antibodies and TNFα levels were quantified by ELISA (n=1).

### 4.4 PGE2 and lipoxin $A_4$

The potential role of PGE2 and lipoxin $A_4$ in the hMSC effects on hMDM inflammatory responses were assessed using specific inhibitors. hMDMs were treated with LPS along with ibuprofen to inhibit PGE2 synthesis or WRW4 to compete for the lipoxin $A_4$ receptor on hMDMs. These inhibitors were also added to hMDMs which were in non-contact co-culture with hMSCs. Ibuprofen had no effect on TNFα production by LPS-treated hMDMs alone (8520.8 vs 8362.1 pg/mL). WRW4 exerted an anti-inflammatory effect on hMDMs given LPS but without hMSC co-culture (8520.8 vs 6188.9 pg/mL). hMSCs reduced TNFα production (8520.8 vs 3259.3 pg/mL) which was unaffected by the addition of ibuprofen (3259.3 vs 3725.8 pg/mL) or WRW4 (3259.3 vs 2988.2 pg/mL) **(Fig 4.2)**. This result suggests that PGE2 and lipoxin $A_4$ are not the mediators of the hMSC anti-inflammatory effect.

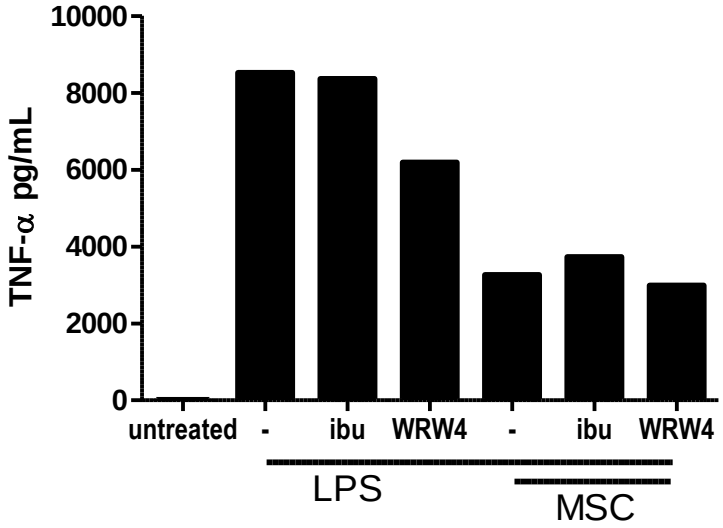



**Fig 4.2 PGE2 and lipoxin $A_4$**

Supernatants were extracted after 24 hours of hMDM stimulation with LPS, with hMSC co-culture and the addition of PGE2 and lipoxin $A_4$ inhibitors**.** TNFα levels were measured (n=1).

### 4.5 Lipocalin-2 and adiponectin

LCN2 and adiponectin production by hMSCs and hMDMs were first quantified. hMDMs and hMSCs were cultured for 24 hours separately and in co-culture with or without LPS stimulation. Supernatants were collected and tested by ELISA for lipocalin-2 and adiponectin. hMDMs produced LCN2 and the extent of secretion was unaffected by LPS treatment (366.7±130.3 vs 345.3±165.2 pg/mL). Surprisingly, hMSCs produced LCN2 at levels barely detectable by the ELISA and this was unchanged with LPS stimulation (15.2±13.0 vs 20.2±15.1 pg/mL). Cells in co-culture with LPS displayed a trend towards a more than additive increase in LCN2 production (513.3±72.7 pg/mL) **(Fig 4.3 A)**. Adiponectin was undetectable by either hMDMs or hMSCs in any condition (data not shown). Given that there was some evidence of increased lipocalin-2 production in co-culture LCN2 function was investigated further. Antibody for LCN2 or isotype was added alongside hMSC-CM to determine whether it would reverse TNFα suppression. hMSC-CM with isotype antibody non-significantly reduced TNFα production (998.0±991.8 vs 282.1±288.2 pg/mL) and this was not prevented with the blockage of LCN2 (282.1±288.2 vs 391.2±401.9 pg/mL) **(Fig 4.3 B)** suggesting that LCN2 is not involved in the hMSC effect. The rejection of this hypothesis was further supported by the observation that hMDMs treated with recombinant human LCN2 produced more IL-8 in response to LPS (17641.1 vs 33778.0 pg/mL) **(Fig 4.3 C)**.

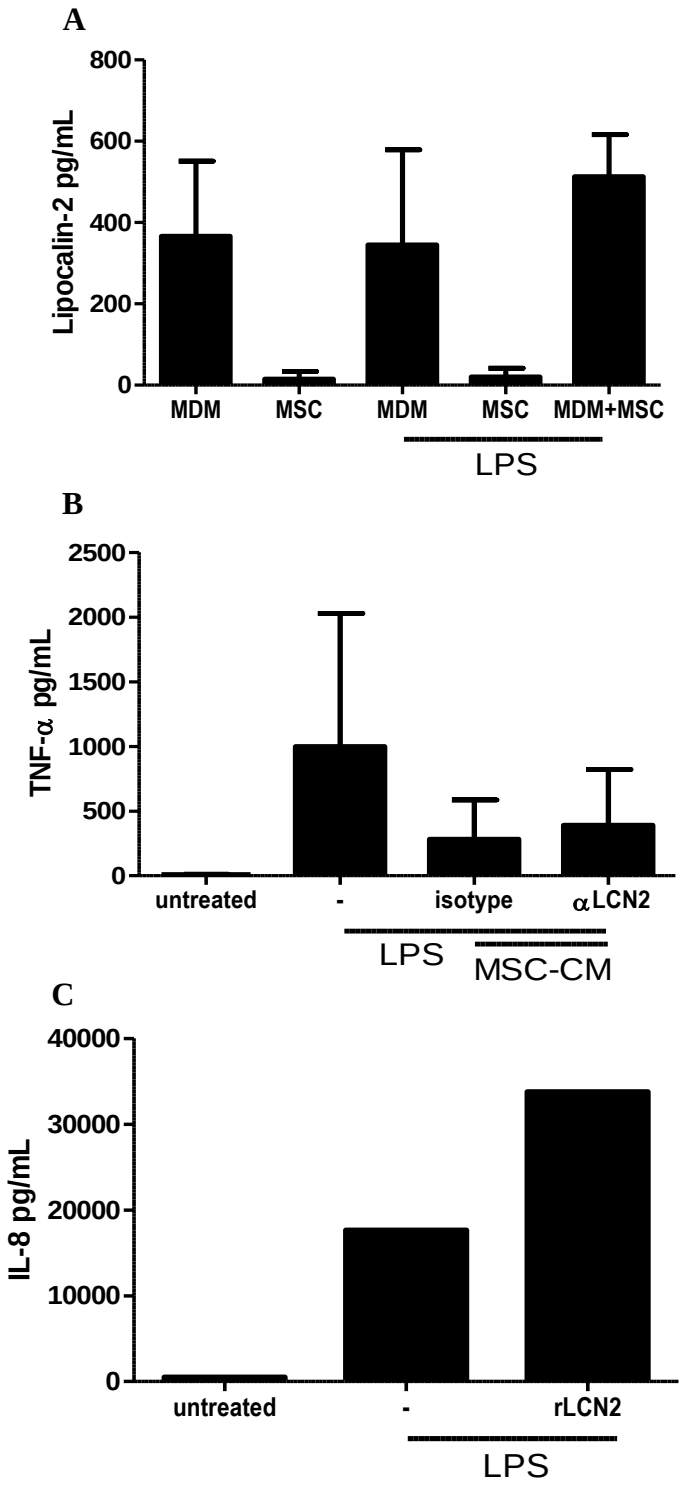



**Fig 4.3 Lipocalin-2**

LCN2 levels were measured in the supernatants of hMSCs and hMDMs with or without LPS (A) (n=2 all groups). LCN2 was neutralised by antibody in hMSC-hMDM co-culture with LPS and TNFα was quantified (B) (n=2 all groups). The effect of recombinant human LCN2 on hMDM IL-8 secretion after LPS treatment was tested (C) (n=1). Error bars represent SD.

### 4.6 Angiopoietin-1

Ang-1 production was determined by ELISA in hMDM and hMSC supernatants with or without LPS. hMDMs produced modest levels of Ang-1 which were not affected by LPS (271.5±28.2 vs 251.0±21.1 pg/mL). hMSCs produced more Ang-1 than hMDMs, but again, LPS did not influence its production (591.3±140.0 vs 548.8±63.7 pg/mL). Surprisingly, hMDM-hMSC co-culture resulted in total Ang-1 levels comparable to hMDMs cultured alone (251.0±21.1 vs 291.4±35.0 pg/mL) **(Fig 4.4)**. This is less than additive and even seems to suggest that hMSC secretion of Ang-1 is switched off by hMDMs. Based on these results, Ang-1 was no longer pursued as a potential candidate.

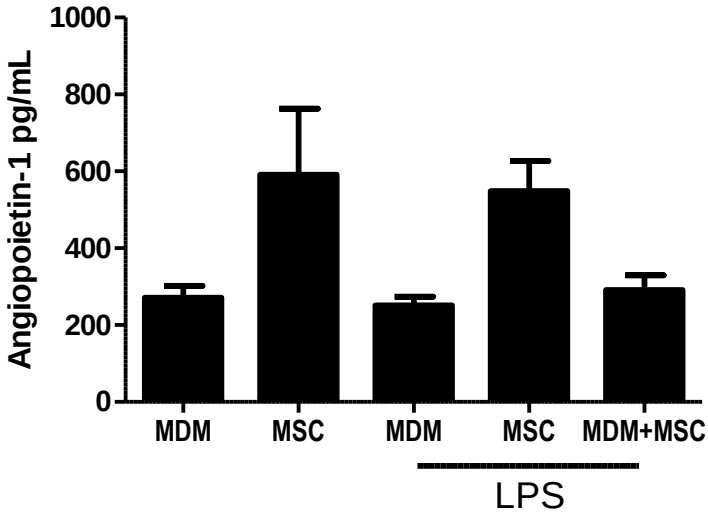



**Fig 4.4 Angiopoietin-1**

hMDMs and hMSCs were cultured for 24 hours with or without LPS stimulation either individually or in non-contact co-culture. Ang-1 secretion was measured in the supernatants by ELISA (n=2). Error bars represent SD.

### 4.7 TIMP3

hMDM and hMSC TIMP3 production was assessed after 24 hours with or without LPS. hMDMs produced very little TIMP3 in the presence and absence of LPS (64.8±8.5 vs 61.9±10.2 pg/mL). hMSCs produced more TIMP3 than the hMDMs and this appeared to be more pronounced after LPS treatment (96.7±8.3 vs 134.1±9.0 pg/mL). Much like Ang-1, TIMP3 production in the co-culture was less than additive and levels mirrored those produced by hMDMs alone (50.2±8.9 pg/mL) **(Fig 4.5)**. Investigation of TIMP3 as a candidate mediator was taken no further.

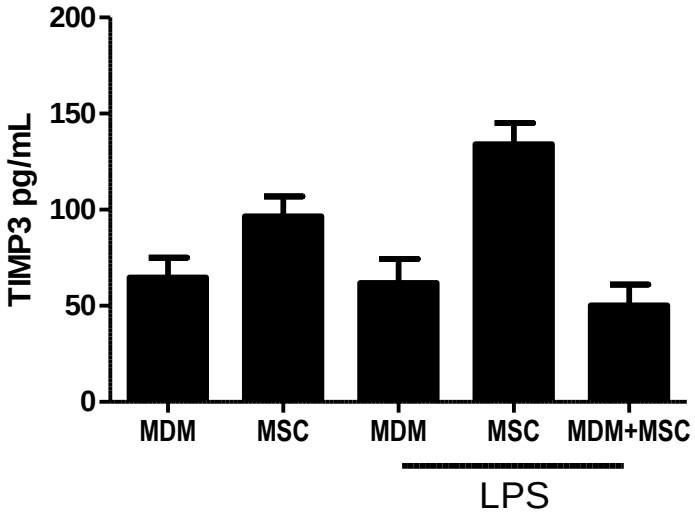



**Fig 4.5 TIMP3**

hMDMs and hMSCs were cultured for 24 hours in the presence or absence of LPS. Supernatants were taken and analysed by TIMP3 ELISA (n=2). Error bars represent SD.

### 4.8 TSG6

hMDMs and hMSCs were then tested for their TSG6 production. A specialised ELISA kit for TSG6 developed by Cusabio was used. hMSCs did not produce any detectable TSG6 either with or without LPS. hMDMs produced massive levels of TSG6 both with LPS and without at concentrations exceeding the top standard of the ELISA at 10ng/mL. This is in spite of diluting these samples by a factor of 30, suggesting that hMDMs were producing TSG6 at concentrations beyond 300ng/mL (data not shown). While accurate quantities cannot be given from these results, the abundance of TSG6 production by hMDMs alone would suggest that hMSCs were not acting through additional TSG6 production. The rationale here was that the factors responsible for the hMSC effect should be produced solely by the hMSCs or at least to a larger degree by the hMSCs.

### 4.9 Discussion

In this chapter a number of soluble mediators which may potentially mediate the effects of hMSCs on hMDMs were considered. IL-6 and TGFβ inhibition had no effect on hMSC suppression of hMDM TNFα production. This was unexpected given reports of the anti-inflammatory capacity of IL-6 and its importance in mediating MSC protective effects in endotoxin-induced lung injury. In this study IL-6-deficient MSCs reduced TNFα expression in injured lungs to a lesser degree[107]. TGFβ also has the capacity to decrease macrophage TNFα expression[472]. However the current study is investigating TNFα production at the protein level as opposed to TNFα mRNA, in addition to the fact that these are human macrophages while these groups were investigating mouse cells. Similarly, neither PGE2 nor lipoxin $A_4$ appear to play a role in the hMSC effect. These factors have both been implicated in exerting anti-inflammatory effects on macrophages. Kure *et al* show reduced TNFα production after lipoxin $A_4$ treatment however this study used a mouse macrophage cell line (RAW264.7 cells)[475]. Nemeth *et al* show increased IL-10 production by macrophages in mice which had received MSCs in a murine sepsis model; this was also associated with decreased TNFα in the serum[85]. The difference in results in the present study are likely a reflection of the vastly different models used; mouse sepsis *in vivo* is not comparable to LPS treatment of hMDMs *in vitro*. The results in this chapter suggest

that hMSCs produce LCN2, Ang-1 and TIMP3 in keeping with the literature[77, 141, 483, 486]. However neither adiponectin nor TSG6 were measurable in these supernatants, even though these are also reportedly produced by MSCs[99, 468, 476, 487]. It was surprising that hMSCs stimulated with LPS would not produce TSG6. TNFα is of course an inducer of TSG6 and whilst this would not be present in the hMSC alone culture, LPS is another inducer of TSG6 as demonstrated in fibroblasts[488]. Because these samples were all above the highest standard in the TSG6 ELISA, it could not be determined whether there was an increase in TSG6 levels with hMSCs in co-culture. It would be plausible in that condition given that they would have been exposed to TNFα. It is likely however that the increase would have been negligible given Lee *et al's* reports of approximately 2.5ng/mL of TSG6 being produced by MSCs with TNFα stimulation, a very small fraction of the >300ng/mL produced by the hMDMs observed here[97]. For that reason, the hypothesis that TSG6 was responsible for the immunomodulatory effects of hMSCs on these hMDMs was rejected. This finding undermines the potential application of quantifying TSG6 production as a potency assay to assess MSC immunomodulatory capacity[155, 489]. Adiponectin was not detectable in either hMSC or hMDM cultures. Abraham *et al* demonstrated increased adiponectin levels in the serum of mice that received bone marrow transplant suggesting that MSCs are contributing to this increase[476]. These were murine MSCs *in vivo* which is not easily comparable to the current study. Winter *et al* show adiponectin gene expression in bone marrow-derived MSCs by RT-PCR rather than protein quantification[487]. Another study suggests that adiponectin production by MSCs only ensues after driving adipogenic differentiation rather than non-committed MSCs[490]. Both Ang-1 and TIMP3 were produced by hMSCs and hMDMs. In single cell cultures hMSCs produced higher levels of these factors than hMDMs. Interestingly, co-culture showed a less than additive quantity of both Ang-1 and TIMP3. It is possible that the paracrine communication between the two cell types resulted in downregulation of expression by either cell type or even both.

LCN2 levels were quite low in hMSC culture whereas hMDMs produced LCN2 to a higher degree. Gupta *et al* were able to demonstrate LCN2 production in MSCs after LPS stimulation but this was in murine cells. They observed increased LCN2 levels in the BALF of mice treated with MSC during *E. coli*-induced pneumonia[77].

Importantly, the increase in LCN2 levels within the BALF was substantially higher than the amount being produced by the MSCs alone; this would suggest that the increased LCN2 is resulting from MSC-induced LCN2 production by other cells. This is in keeping with the results from the current study where co-culture of hMSCs and hMDMs results in increased LCN2 production. It may be that the hMSCs have increased hMDM LCN2 secretion. In this case LCN2 would not be the hMSC-derived factor mediating their effect on hMDMs, rather it would be released by hMDMs in response to that factor. Induction of LCN2 production by hMDMs may simply represent the acquisition of heightened antimicrobial capacity due to hMSCs or it may also serve to further promote induction of the hMSC-induced M2-like macrophage phenotype in an autocrine manner. The role of LCN2 in LPS-induced hMDM TNFα production was studied. Blockage of LCN2 by antibody neutralisation did not abrogate hMSCs ability to suppress TNFα production and recombinant LCN2 even augmented the IL-8 response with LPS stimulation. Zhang *et al* demonstrated that 500ng/mL of recombinant LCN2 reduced macrophage TNFα production[398]. This is a much higher level than those observed in my *in vitro* cultures which may not be sufficient to mitigate the hMDM inflammatory response.

### 4.10 Summary and conclusions

Based on the results in this chapter, a number of soluble mediators suggested by the literature were disregarded as potential candidates responsible for the hMSC effect. So far, the mechanisms of hMSC effect on hMDM phenotype and function in this model remain unknown. Results from this chapter suggest that:

- IL-6, TGFβ, PGE2 and lipoxin $A_4$ do not facilitate the anti-inflammatory effects of hMSCs on hMDMs
- Adiponectin is not produced by hMSCs with or without LPS stimulation
- Lipocalin-2 is produced to a larger degree by hMDMs but plays no role in their modulation by hMSCs
- Ang-1 and TIMP3 are produced by hMSCs and hMDMs but seem to be downregulated in co-culture suggesting that they do not play a role
- TSG6 is produced by hMDMs but not substantially by hMSCs

# Chapter 5

# Investigation of mechanisms of macrophage modulation mediated by hMSC-EVs

## 5.1 Introduction

Having tested a range of different paracrine factors which were shown to play no role in the hMSC effects observed on hMDMs, the next candidate to investigate was hMSC-derived EVs. Described in detail in **Chapter 1**, EVs are small membrane-bound compartments produced by cells which may contain a plethora of soluble factors including proteins, miRNA and even organelles[291, 295]. These vesicles provide another means of cellular communication and have key roles in cellular processes including coagulation, angiogenesis and metastasis[301, 491, 492]. MSCs are known to have a high capacity for EV secretion[493] and these have been shown to be responsible for a number of their beneficial effects in preclinical models investigating tumour growth, airway inflammation and pulmonary hypertension amongst others[166, 168, 170]. This is also the case in lung injury models; a number of studies have provided evidence that MSC-EVs are able to improve survival, reduce lung injury, measured by the extent of protein-rich pulmonary oedema, reduce inflammation based on neutrophil influx and cytokine levels and improve bacterial clearance though the augmentation of monocyte phagocytosis[171, 317]. Importantly Monsel *et al* highlight the importance of CD44 expression on MSC-EVs for their effective uptake by primary human monocytes and alveolar epithelial cells[171]. Researchers are now investigating the potential of using EVs therapeutically, instead of whole cell therapies[175, 322, 494].

MSC-EVs are known to contain KGF mRNA, which is partly responsible for their therapeutic effects in lung injury models[317]. Microarrays have shown that these EVs also contain a multitude of miRNAs which could regulate a host of other processes. Intriguingly, the miRNA content of MSCs and their EVs differ considerably, suggesting that there is active sequestration of specific miRNAs into these exosomal compartments[291]. These MSC-derived miRNAs were found to exert anti-inflammatory effects on macrophages via the downregulation of TLR signalling. Indeed, MSC-EV miRNAs have been demonstrated to mediate a number of their regulatory functions. miR-133b found in MSC-EVs stimulates neurite growth and miR-31 was able to promote angiogenesis in endothelial cells by targeting and inhibiting factor-inhibiting HIF-1[320, 321]. Perhaps most impressively, MSC-EVs can also contain functional mitochondria which are transferrable to recipient cells,

evidenced by Phinney *et al*, where they studied mitochondrial transfer to macrophages[291]. Mitochondrial transfer has also been reported through contact-dependent mechanisms such as tunnelling nanotubules and gap junction-assisted EV transfer[116, 318]. This exchange of mitochondria can have a number of effects on the recipient cell; these studies have reported enhancement in cellular ATP levels as well as promoting phagocytic activity. The acquisition of functional mitochondria, particularly from another cell type, could have more substantial effects on cellular homeostasis than those already reported. It is unsurprising that there is a resultant increase in ATP production in alveolar epithelial cells receiving MSC-derived mitochondria, given they are the powerhouses of the cell. Mitochondria, however, play key roles in other important cellular processes. They may initiate programmed cell death through the intrinsic pathway and mitochondrial ROS generation is important for bactericidal function of phagocytes[359, 363]. In fact, there is more and more emerging evidence showcasing the involvement of mitochondria in innate immune functions. Mitochondria play a pivotal role in anti-viral responses mediated by the RIG-1/MDA-5/MAVS signalling pathway leading to type I IFN production and both IRF3 and NF-κB activation[370]. They are capable of NLRP3 inflammasome activation through ROS production[371]. Mitochondria facilitate so many central functions that it seems feasible that mitochondrial transfer has consequences more complex than simply increasing cellular metabolic capacity.

Macrophage polarisation and metabolism are integrally linked. The metabolism of iron and arginine is indicative of macrophage activation state[248, 253, 255]. M1 macrophages activated by LPS or IFNγ utilise primarily glycolysis, whereas M2 macrophages are characterised by a switch to fatty acid oxidation and mitochondrial respiration[239, 240]. Vats *et al* showed that alternative activation of murine macrophages induced by IL-4 was dependent on mitochondrial respiration[240]. The inhibition of mitochondrial respiration using either oligomycin or FCCP resulted in perturbed IL-4-dependent M2 activation measured by reduced arginase activity. Furthermore, these inhibitors also sustained TNFα, IL-6 and IL-12p40 production in response to LPS/IFNγ, blocking the anti-inflammatory effects of additional IL-4 treatment. The mitochondria's pivotal role in regulating metabolic activity as well as the strong association between macrophage activation and glycolysis/oxidative phosphorylation

balance, leads to the hypothesis that mitochondrial transfer influences macrophage polarisation.

## 5.2 Aims

In **Chapter 3**, the hMDM phenotype that is promoted by hMSCs was characterised *in vitro*. In the current chapter there were three main aims:

- Investigate the role of hMSC-EVs in hMDM phenotype modulation
- Determine the importance of hMSC-EV modulation of macrophages *in vivo*
- Investigate the roles of EV-derived miRNAs and mitochondria in the hMSC effect on macrophage phenotype

## 5.3 hMSC-derived EVs are present in hMSC-CM and are characterised by high expression of CD44 on their surface

To test the hypothesis that hMSC-EVs play a role in the modulation of macrophage phenotype by hMSCs, it was important to confirm that EVs are present in these hMSC-CM preparations. Firstly, to provide some indication of the size of hMSC-EVs, hMSC-CM was centrifuged to remove cell debris and then analysed by flow cytometry alongside latex beads which have a diameter of 4μm. Latex beads alone were first gated for by FSC vs SSC. Latex beads were then added to hMSC-CM and analysed at the same settings. The EV population was situated below the latex beads on the FSC vs SSC plot indicating that EVs are considerably smaller **(Fig 5.1 A)**. hMSCs were grown to confluence before being stained with a fluorescent anti-CD44 antibody (or isotype control) and a general cell membrane stain (cell mask). hMSCs were also stained with propidium iodide and annexin V to determine the amount of necrotic or apoptotic cell bodies respectively. hMSCs were washed and CM was generated for 24 hours in the presence of LPS stimulation. Cell debris was removed by centrifugation before proceeding to flow cytometry of EVs within the CM. To identify the EV population, the following gating strategy was used: FSC vs SSC showed a dense population of EVs at the limits of sensitivity of the cytometer. Of all events 96.1% were cell mask positive and were therefore cell membrane-derived. Propidium iodide and annexin V stains showed that there was small contamination with necrotic (2.4%)

and apoptotic (9.1%) debris in this EV population. Events which were propidium iodide and annexin V negative were gated for. The gate for CD44 staining was adjusted according to the isotype control and 94.8% of EVs were found to express CD44 **(Fig 5.1 B)**.

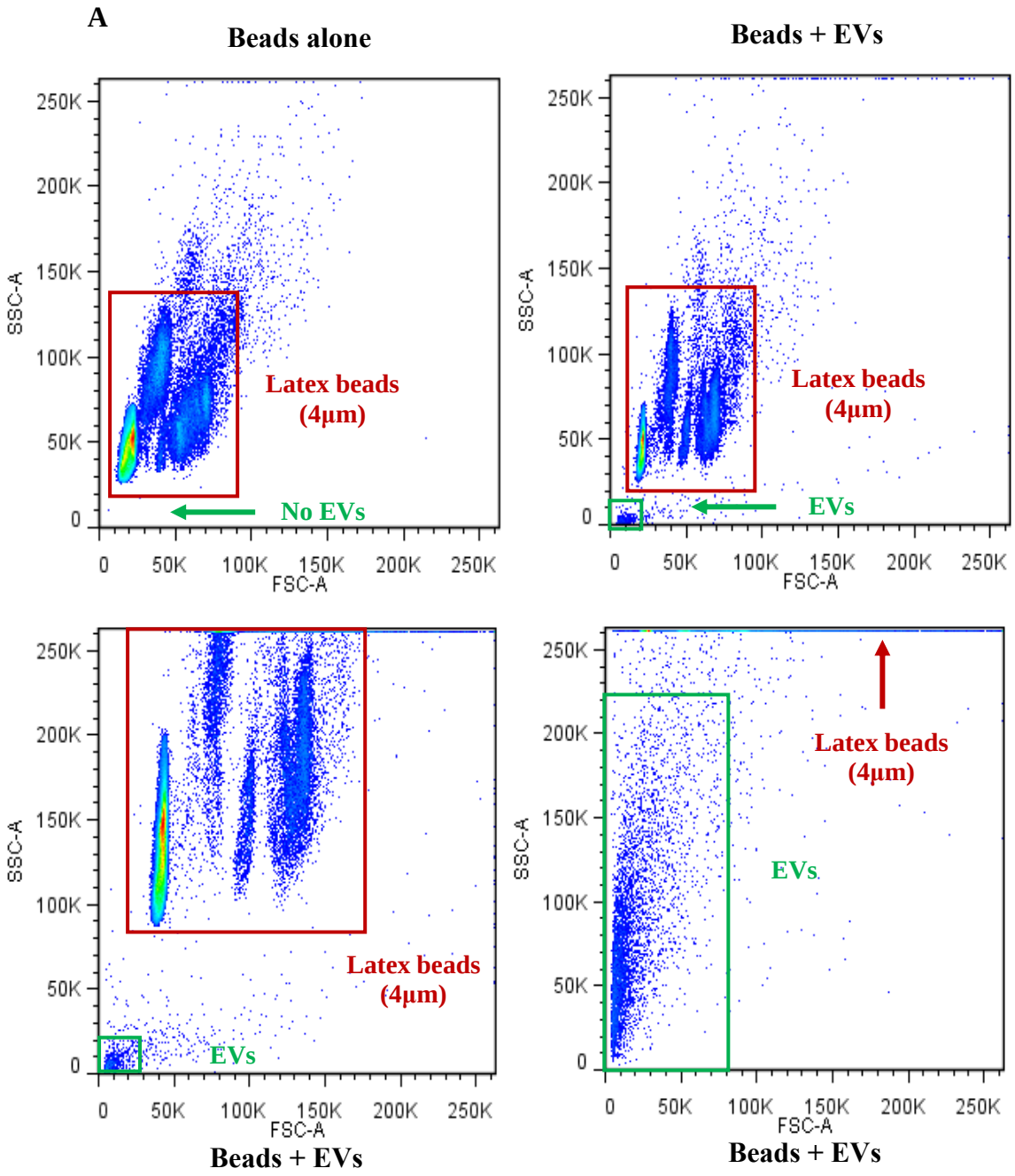



**Fig 5.1 hMSC-derived EVs express CD44 on their surface (continued overleaf)**

hMSC-CM-derived EVs were analysed by flow cytometry alongside latex beads of known size to provide a reference point (A). FSC and SSC voltage settings were progressively increased to focus in on the EVs.

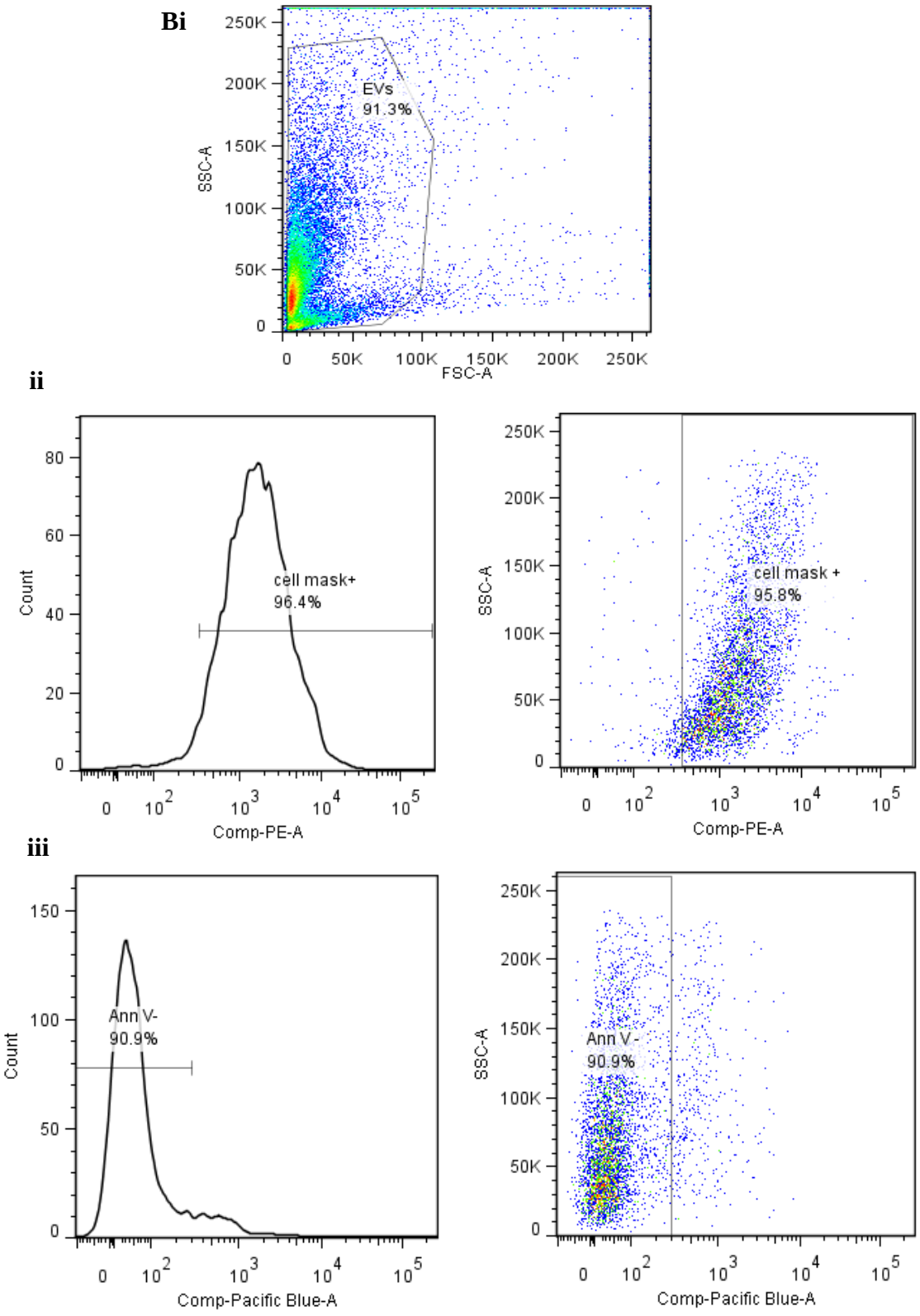



**Fig 5.1 hMSC-derived EVs express CD44 on their surface (gating strategy continued overleaf)**

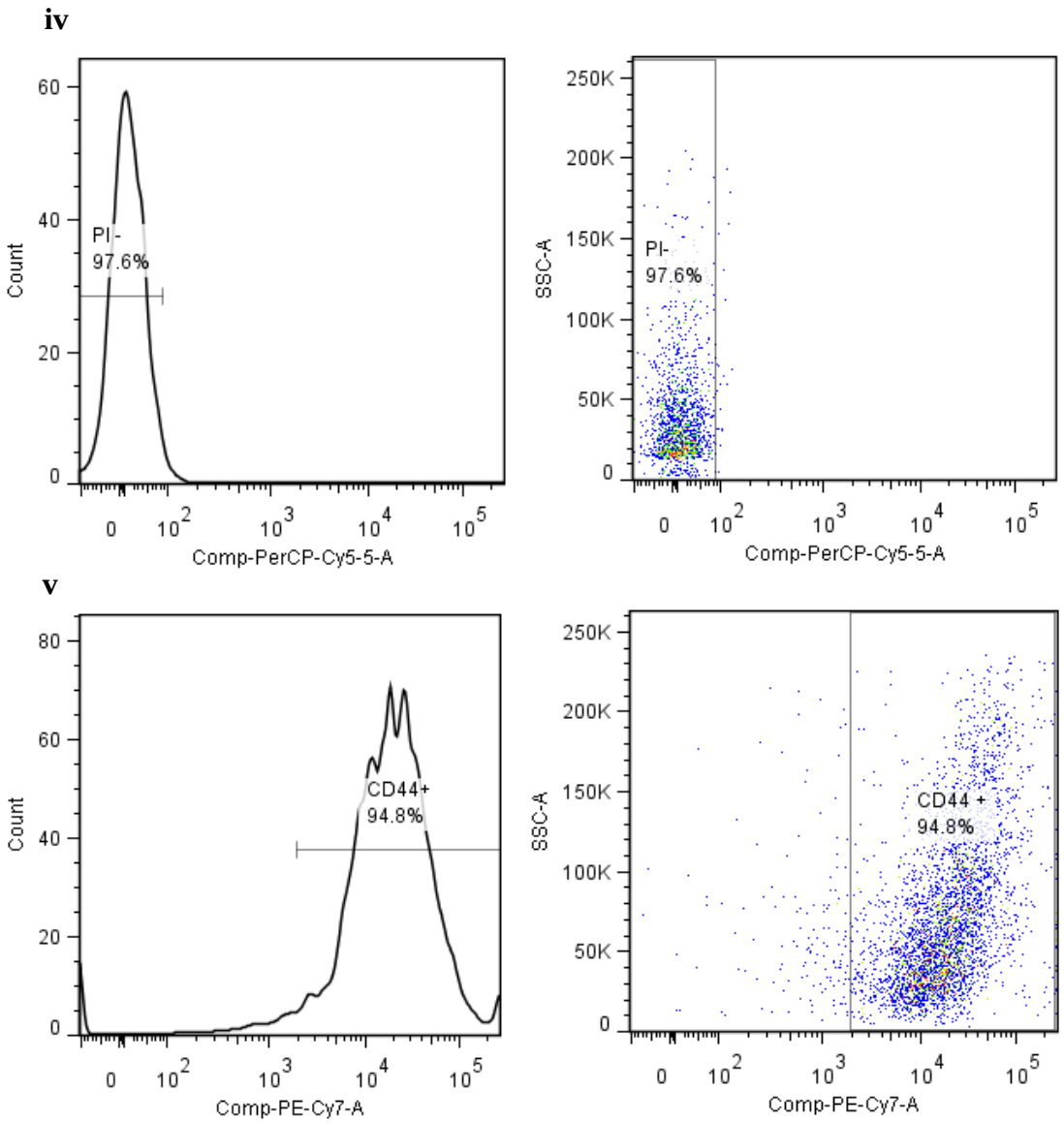



**Fig 5.1 hMSC-derived EVs express CD44 on their surface**

MSCs were pre-stained with propidium iodide, annexin V, cell mask and anti-CD44 antibody or isotype control before generating CM. CM was centrifuged to remove cell debris and was then analysed by flow cytometry. EVs were selected by FSC vs SSC (i), cell mask positive events were gated for (ii), apoptosis contamination was accounted for (annexin V, Ann V) (iii), then necrosis (propidium iodide, PI) was ruled out (iv) and finally CD44 positivity was determined (v). Results representative of two independent experiments.

## 5.4 hMSC-derived CD44-expressing EVs are partially responsible for suppression of LPS-induced TNFα secretion by hMDMs

To determine if these CD44-expressing EVs were important for hMSC effects, CM from hMSCs was prepared as before and pre-incubated with a neutralising antibody for CD44 or isotype control. hMDMs were then stimulated with LPS and hMSC-CM with or without antibody pre-treatment. hMDMs treated with LPS and antibody, but not hMSC-CM, were included as a control for the effects of the antibodies on hMDMs. Supernatants were lifted and TNFα production was determined by ELISA. Treatment of hMDMs with anti-CD44 had no effect on TNFα levels and isotype had no effect in LPS or LPS + hMSC-CM-treated hMDMs. Consistent with previous data, hMSC-CM significantly reduced LPS-induced production of TNFα by macrophages (1312.0±229.4 vs 389.6±255.7 pg/mL) ($p<0.05$). Pre-incubation of hMSC-CM with anti-CD44 resulted in partial abrogation of this effect, suggesting that CD44 expressing EVs present in the hMSC-CM are at least partially responsible for the MSC effect **(Fig 5.2)**.

## 5.5 hMSC-derived CD44-expressing EVs are critical for enhancement of hMDMs phagocytic activity

Next the importance of CD44-expressing EVs in the enhancement of hMDM phagocytosis was investigated. Using the same conditions, for 24 hours of stimulation, supernatants were aspirated and the hMDMs were washed before adding *E. coli* bioparticles to assess phagocytic activity. hMSC-CM increased the proportion of phagocytic hMDMs by 28.1% ($p<0.01$), and produced a 9-fold increase in MFI (360.8 vs 3249.8) ($p<0.01$), in the presence of LPS. Pre-incubation of hMSC-CM with anti-CD44 completely reversed the enhancement in phagocytosis by both percentage (80.4±4.8 vs 61.1±7.6 %) ($p<0.05$) and MFI (3249.8±2240.2 vs 753.8±529.8). Isotype control had no influence on hMDM phagocytosis or the hMSC effect **(Fig 5.3)**.

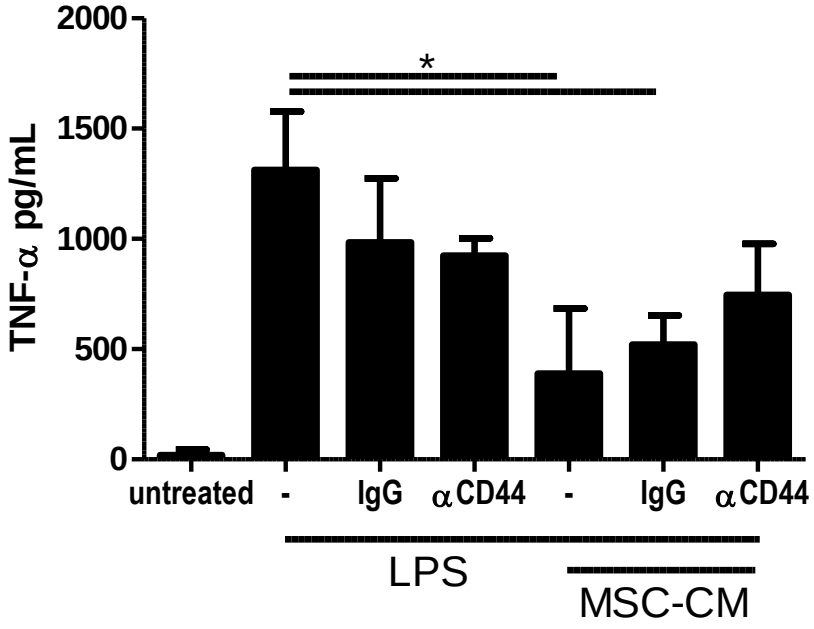



**Fig 5.2 hMSC-derived CD44-expressing EVs are partially responsible for suppression of LPS-induced TNFα secretion by hMDMs**

hMDMs were treated with LPS for 24 hours with hMSC-CM which had been pre-incubated with or without anti-CD44 (or isotype). Supernatants were taken for analysis of TNFα production. Error bars represent SD (n=4 all groups) (Kruskal Wallis with Dunn's post hoc test, *$p<0.05$)

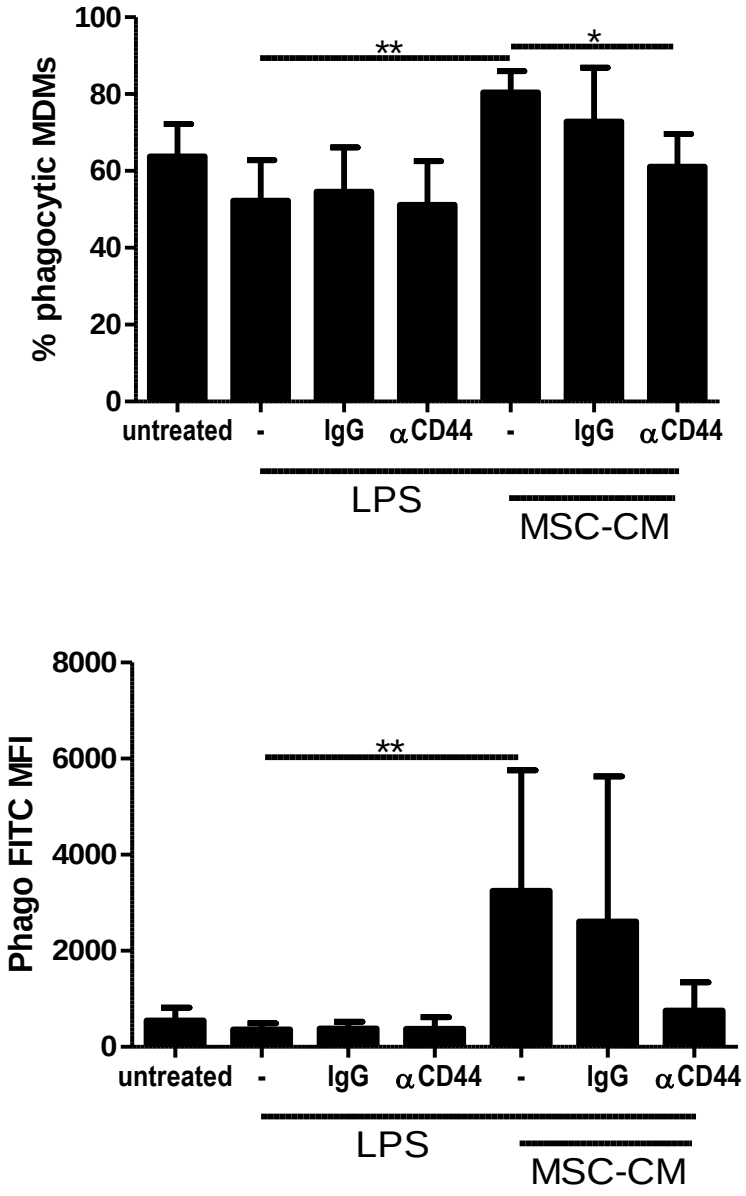



**Fig 5.3 hMSC-derived CD44-expressing EVs are critical for the enhancement of hMDMs phagocytic activity**

hMDMs were treated with LPS for 24 hours with hMSC-CM which had been pre-incubated with or without anti-CD44 (or isotype). E. coli bioparticles were used to determine phagocytic activity and is presented as percentage phagocytic cells (A) and MFI (B). Error bars represent SD (n=5 all groups) (Kruskal Wallis with Dunn's post hoc test, *$p<0.05$, **$p<0.01$).

## 5.6 AMs are the cellular mediators of hMSC-EVs beneficial effects in *E. coli* endotoxin-induced lung injury *in vivo*

After establishing that EVs were primarily responsible for the paracrine mechanism of hMSC modulation of macrophage phenotype *in vitro*, it was necessary to test the importance of hMSC-educated macrophages in lung injury *in vivo*. C57BL/6 male mice at eight weeks of age were culled for BAL to isolate AMs. EVs were isolated from hMSC-CM and these were added to the murine AMs *ex vivo*. AMs cultured *ex vivo* without hMSC-EVs were included as a control. Mice were injured with LPS or PBS and four hours later treated with AMs which had received EV treatment, AMs without treatment or PBS. 24 hours after injury, mice were culled for BAL. BALF was processed for total cell counts, neutrophil counts and protein content. The experiment was performed twice; once treating mice with untreated AM and the other treating mice with AM that had been cultured with hMSC-EVs. These experiments were performed separately due to the difficulty in isolating sufficient numbers of AM from mice by BAL for *ex vivo* culture. Cytospins prepared from the BALF of these mice were imaged at 20x magnification **(Fig 5.4 A)**. In the first experiment LPS administration produced a substantial influx of inflammatory cells into the airspaces compared to PBS-treated mice (0.29±0.10 vs 1.18±0.34 $x10^6$ total cells/mL) which included a large proportion of neutrophils (<0.01 vs 0.38±0.18 $x10^6$ neutrophils/mL). Untreated AMs did not influence these cell numbers (1.18±0.34 vs 1.14±0.22 $x10^6$ cells/mL) (0.38±0.18 vs 0.48±0.10 $x10^6$ neutrophils/mL) **(Fig 5.4 B)**. Consistent with inflammatory cell infiltration, LPS treatment resulted in an increase in BALF protein levels compared to the PBS group (295.87±27.45 vs 569.04±324.19 μg/mL). This indicates protein rich oedema formation due to impairment of epithelial-endothelial barrier integrity. Untreated AMs were unable to reduce protein content in the BALF compared to LPS-injured mice receiving PBS (569.04±324.19 vs 593.67±85.17 μg/mL) **(Fig 5.4 C)**. In the second experiment LPS produced a more robust inflammatory response, evidenced by higher total cell counts as well as absolute neutrophil counts in the BALF (0.29±0.10 vs 1.95±0.26 $x10^6$ cells/mL) (<0.01 vs 1.41±0.39 $x10^6$ neutrophils/mL). EV-treated AMs significantly reduced both total cell counts and neutrophilia in the BALF (1.95±0.26 vs 1.08±0.25 $x10^6$ cells/mL) ($p<0.01$) (1.41±0.39 vs 0.54±0.14 $x10^6$ neutrophils/mL) ($p<0.05$) **(Fig 5.4 D)**. LPS instillation induced lung injury as indicated by an increase in BALF protein levels compared to

uninjured controls (295.87±27.45 vs 674.41±64.22 μg/mL). Importantly, EV-treated AMs were able to prevent this lung injury, reducing BALF protein to levels comparable to the uninjured mice (674.41±64.22 vs 326.13±38.70 μg/mL) ($p<0.001$) **(Fig 5.4 E)**. This study demonstrates that hMSC-EVs are capable of macrophage modulation that is critical in providing protection from lung injury *in vivo* and also highlights the role of AMs as important cellular mediators of the hMSC effect.

A

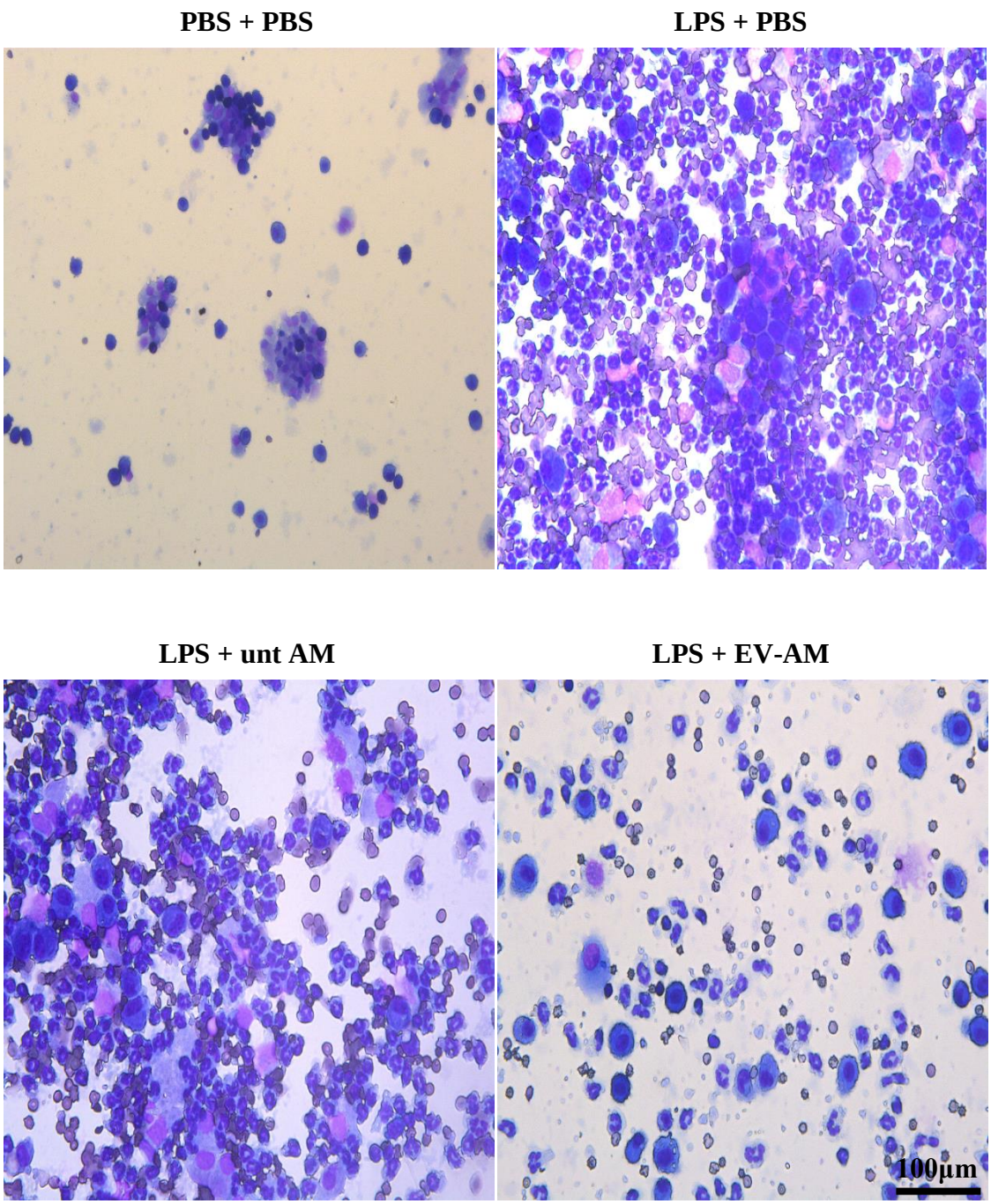



**Fig 5.4 AMs are the cellular mediators of hMSC-EVs beneficial effects in *E. coli* endotoxin-induced lung injury *in vivo* (continued overleaf)**

Mice were injured with LPS administration and treated with PBS, or AMs which themselves were untreated or cultured with hMSC-EVs. 24 hours after injury, mouse lungs were lavaged. Cytospins were prepared from the BALF, cells were stained and imaged by brightfield microscopy (20x magnification). Representative images for each group are shown (A).

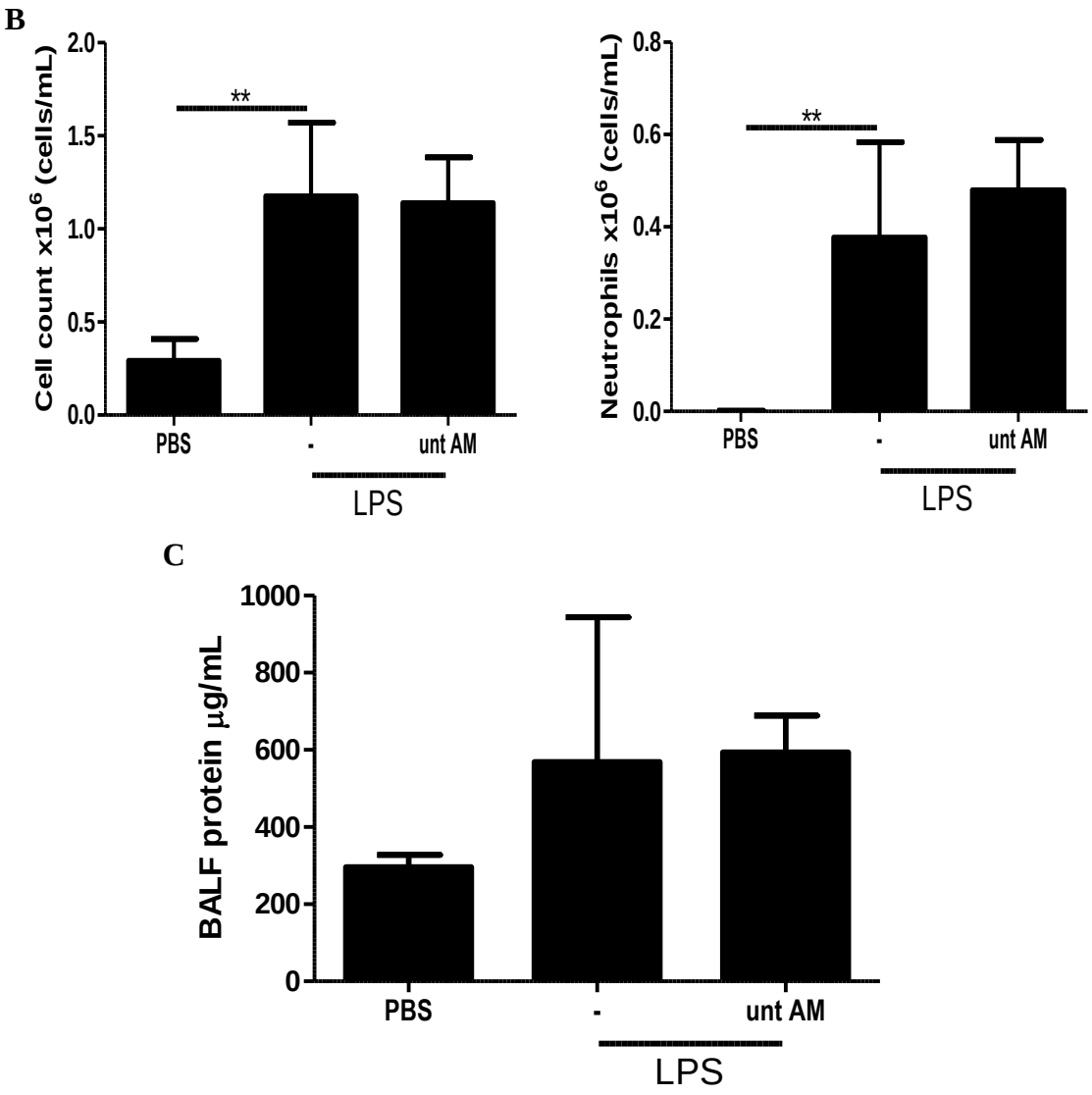



**Fig 5.4 (continued) AMs are the cellular mediators of hMSC-EVs beneficial effects in *E. coli* endotoxin-induced lung injury *in vivo* (continued overleaf)**

Prior to cytospin preparation, aliquots were taken from BALF samples and total cell counts performed. Differential counts of cytospin images were performed to quantify neutrophils (B). Protein content of cell-free BALF supernatant was quantified by BCA (C). Results from the untreated AM-treated mice are shown. Error bars represent SD (n=4 all groups but untreated AM group which is n=5) (One way ANOVA with Bonferroni's post hoc test, **$p<0.01$).

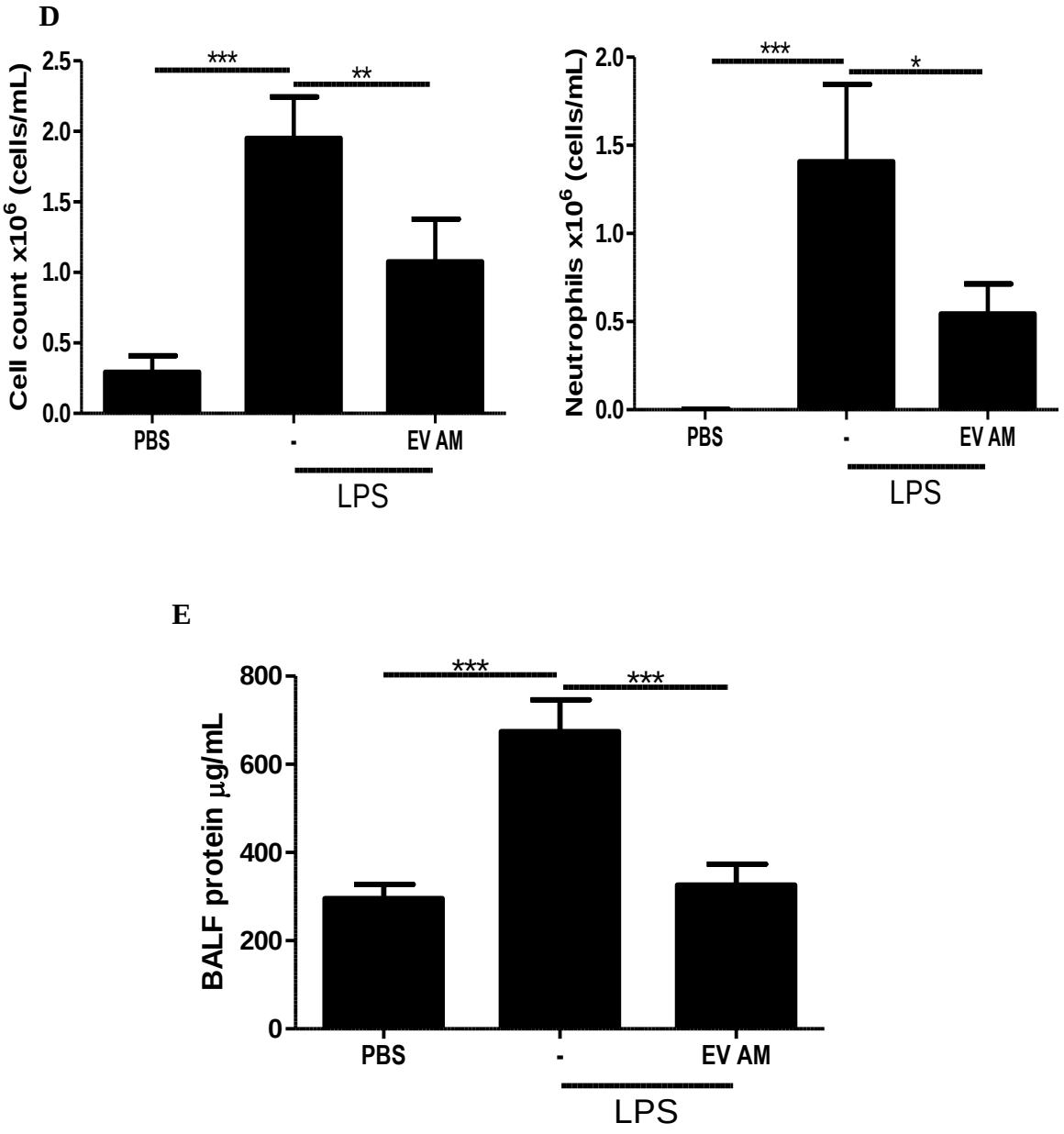



**Fig 5.4 (continued) AMs are the cellular mediators of hMSC-EVs beneficial effects in *E. coli* endotoxin-induced lung injury *in vivo***

Prior to cytospin preparation, aliquots were taken from BALF samples and total cell counts performed. Differential counts of cytospin images were performed to quantify neutrophils (D). Protein content of cell-free BALF supernatant was quantified by BCA (E). Results from the EV-treated AM-treated mice are shown. Error bars represent SD (n=4 PBS group, n=5 LPS group, n=3 EV AM group) (One way ANOVA with Bonferroni's post hoc test, *p<0.05, **p<0.01, ***p<0.001).

## 5.7 Investigation of the role of hMSC-EV-derived miRNAs in the mechanisms underpinning the hMSC-EV effect on hMDMs

Having established the important role of hMSC-EVs in mediating hMSC modulation of macrophage function, the next step was to begin investigation of the EV contents responsible for the effect. Phinney *et al* were the first to demonstrate that miRNA-containing MSC-EVs had an anti-inflammatory effect on macrophages; it was observed that the removal of miRNA from the MSC-EV compartment by inhibiting DICER abrogated their ability to modulate TLR expression. Therefore it was hypothesised that the anti-inflammatory effects of hMSC-EVs in the present study are mediated by miRNAs. Phinney *et al* have performed a miRNA screen on both MSCs and their EVs to determine their content[291], they did not however investigate the roles of individual miRNAs. Microarray data have been deposited in the GEO NCBI database (accession code GSE71241); analysis of these data identified several specific miRNAs as promising candidates to be tested for their involvement in the hMSC-EV effect on macrophages in the present study. As reported by Phinney *et al* miR-125b was amongst those present in both MSCs and their EVs; this miRNA has a number of roles in regulating myeloid cell function. miR-125b targets TNFα mRNA transcripts in macrophages inhibiting its translation[495]. This miRNA also stabilises κB-Ras2, an inhibitor of NF-κB, thereby inhibiting pro-inflammatory cytokine secretion[496]. miR-125a is produced by MSCs and acts to suppress M1 macrophage M1 activation while promoting M2 activation[497, 498]. Another candidate for the hMSC effect is miR-223 which also dampens pro-inflammatory activity in macrophages by targeting Pknox1[347, 499]. I sought to determine whether miR-125a, miR-125b or miR-223 were responsible for the hMSC effect on hMDMs. miRNA was extracted from hMSCs and qPCR was performed to determine whether these miRNAs were present. qPCR confirmed the presence of miR-125a and miR-125b and while miR-223 was detected the CT values were very high **(Fig 5.5 A)**. Next miRNA was extracted from hMSCs-EVs and the experiment repeated. miR-125b was the only miRNA present in considerable abundance based on CT values although the gradient of the amplification plots for miR-125a and miR-223 suggest some inhibition of the PCR reaction **(Fig 5.5 B)**. In order to investigate the functional importance of miR-125a and miR-125b, hMSCs were transfected with anti-miRNA inhibitors for these targets using oligofectamine (Thermo Fisher Scientific). A non-specific negative control inhibitor

was included as a control for the transfection process. The efficacy of the inhibitors was first confirmed by qPCR; use of anti-miR-125b completely abolished miR-125b expression in hMSCs and it was no longer detectable. Similarly anti-miR-125a completely blocked miR-125a expression, however there appeared to be some non-specific inhibition of miR-125a with the anti-miR-125b inhibitor and vice versa **(Fig 5.5 C)**. hMSCs used in stimulation experiments were treated with the anti-miR-125b inhibitor but considered to have ablated miR-125a and miR-125b expression. CM from transfected hMSCs was taken and added to hMDMs treated with LPS for 24 hours. Supernatants were analysed by ELISA for TNFα production. LPS produced a robust TNFα response which was reduced by CM from hMSCs that had been transfected with either negative control or anti-miR-125b inhibitors. This suggests that miR-125b is not responsible for the anti-inflammatory effect of hMSC-CM **(Fig 5.5 D)**. hMDMs were washed after isolating the supernatants and *E. coli* bioparticles were added to evaluate phagocytosis. hMSC-CM from negative control inhibitor transfected cells produced a consistent increase in phagocytosis by both percentage of phagocytic hMDMs and MFI, however the use of specific anti-miR-125b inhibitor did not affect this, again suggesting that miR-125b is not involved in hMSC-CM enhancement of phagocytosis **(Fig 5.5 E)**.

**A**

| hMSCs | |
|---|---|
| **miRNA** | **CT value** |
| miR-125a | 20.82 |
| miR-125b | 16.32 |
| miR-223 | 39.87 |

**B**

| hMSC-MVs | |
|---|---|
| **miRNA** | **CT value** |
| miR-125a | 39.10 |
| miR-125b | 26.77 |
| miR-223 | 36.28 |

**C**

| hMSC transfection | | |
|---|---|---|
| **Treatment** | **miRNA** | **CT value** |
| No treatment | miR-125a<br>miR-125b | 32.77<br>22.09 |
| Negative control inhibitor | miR-125a<br>miR-125b | 35.34<br>23.09 |
| anti-miR-125a | miR-125a<br>miR-125b | No CT<br>40.485 |
| anti-miR-125b | miR-125a<br>miR-125b | No CT<br>49.39 |

**Fig 5.5 hMSC-EV-derived miR-125a and miR-125b are not responsible for the hMSC effect on hMDMs (continued overleaf)**

miRNA from hMSCs and their EVs were analysed by qPCR for detection of miR-125a, miR-125b and miR-223 (A and B respectively) (n=1). Efficacy of miR-125a and miR-125b inhibition using transfection was determined by qPCR (C) (n=1). qPCR results are presented using raw data amplification plots and average CT values.

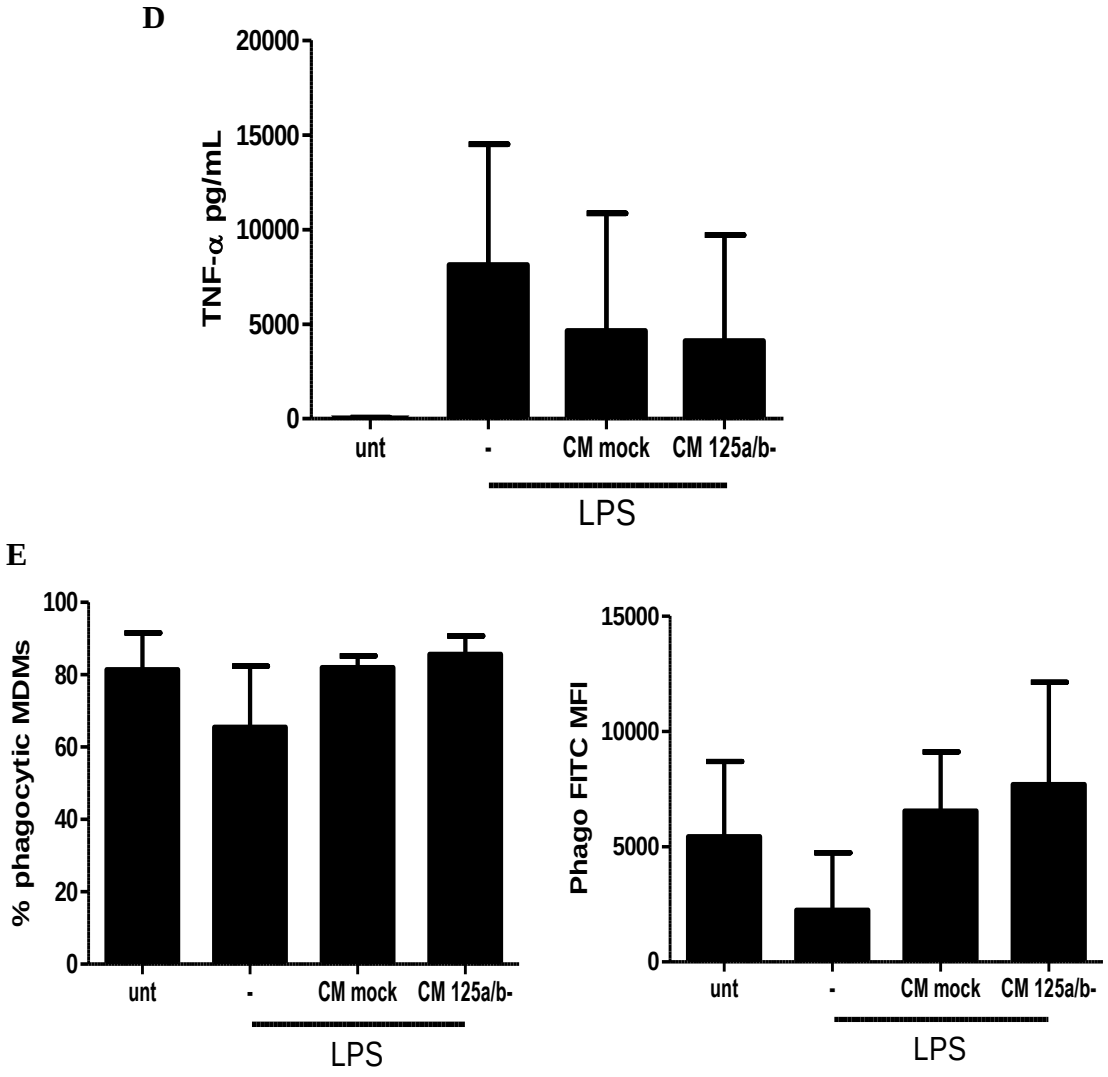



**Fig 5.5 (continued) hMSC-EV-derived miR-125a and miR-125b are not responsible for the hMSC effect on hMDMs**

CM from hMSCs which had been transfected with the negative control and miR-125a/b inhibitors were added to LPS-treated hMDMs; their supernatants were tested for TNFα by ELISA (D) and *E. coli* bioparticles were used to quantify phagocytosis (E). Percentage phagocytic hMDMs and MFI for phagocytic index are presented. Error bars represent SD (n=4 all groups).

### 5.8 hMSC-EVs contain mitochondria which are transferred to hMDMs

Our group has recently published a paper showing that hMSCs can donate their mitochondria to hMDMs through tunnelling nanotubules which mediates the enhancement of macrophage phagocytosis[116]. MSC-EVs are known to contain mitochondria which is another means of transferring these organelles to other cell types[291, 318]. I hypothesised that transfer of mitochondria via EVs is responsible for the hMSC effect on hMDMs. To test this hypothesis it was important to first confirm that mitochondria-containing EVs are present in these hMSC-CM preparations. When preparing the hMSCs for flow cytometry as described in **Section 5.3**, they were also pre-stained with MitoTracker Red; a highly specific dye for functional mitochondria[500, 501]. Using the same gating strategy outlined in **Section 5.3**, the amount of EVs containing mitochondria was quantified. The proportion of total EVs containing mitochondria was 25.2% **(Fig 5.6 A)**. To test for EV-mediated mitochondrial transfer, hMDMs were cultured on chamber slides and pre-stained with MitoTracker Green and Hoechst nuclear stain (blue). hMSCs were pre-stained with MitoTracker Red and CM was produced after extensive washing for 24 hours. hMSC-CM was added to the pre-stained hMDMs and cultured for a further 24 hours. Uptake of hMSC mitochondria into hMDMs was visualised by fluorescence microscopy (x40 magnification). Evidence of mitochondria-containing EV adhesion to hMDMs is evident with red hMSC mitochondria visible on the green hMDM mitochondria (white arrows). Integration of hMSC mitochondria into the hMDM mitochondrial network is apparent where the red and green fluorescence co-localise to give a yellow hue (yellow arrow) **(Fig 5.6 B)**. Finally, MitoTracker Red pre-stained hMSCs in non-contact co-culture or their CM was added to unstained hMDMs in the presence of LPS. The hMDMs were processed by flow cytometry to determine whether they acquired MitoTracker Red fluorescence. 21.7% of hMDMs in non-contact co-culture with hMSCs acquired hMSC mitochondria and this was further increased to 55.1% with the use of hMSC-CM ($p<0.01$) **(Fig 5.6 C). NB: Fig 5.6 C data features in Figure 7 of our group's recently published paper (Jackson *et al*, 2016)[116].**

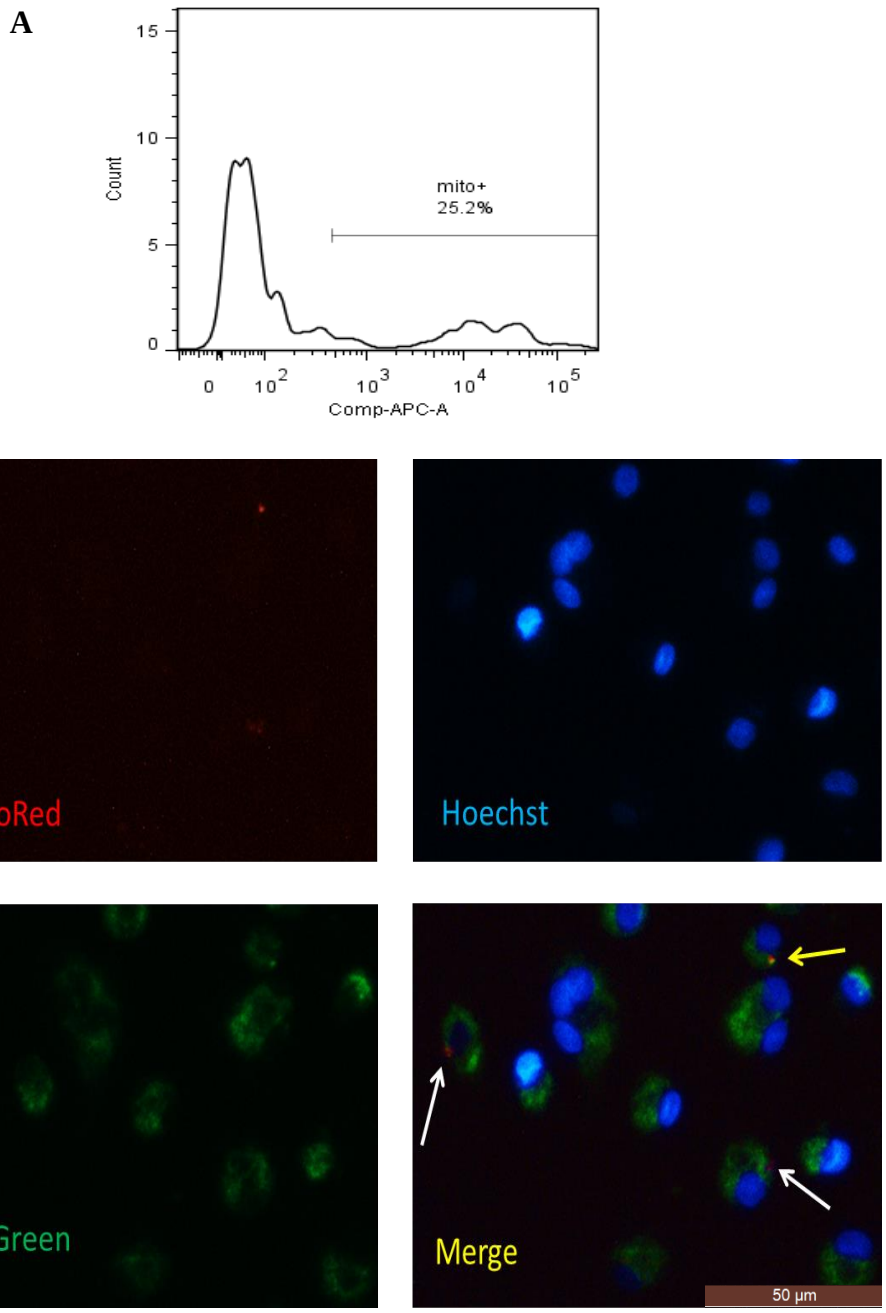



**Fig 5.6 hMSC-EVs contain mitochondria which are transferred to hMDMs (continued overleaf)**

hMSCs were pre-stained with MitoTracker Red and EVs were selected using the gating strategy described in **Section 5.3**. hMSC-CM was taken and analysed by flow cytometry to determine mitochondrial content (A). hMDMs were stained with MitoTracker Green and Hoechst nuclear stain. CM from MitoTracker Red stained hMSCs was added to hMDMs and mitochondrial transfer was visualised by fluorescence microscopy at 20x magnification (B).

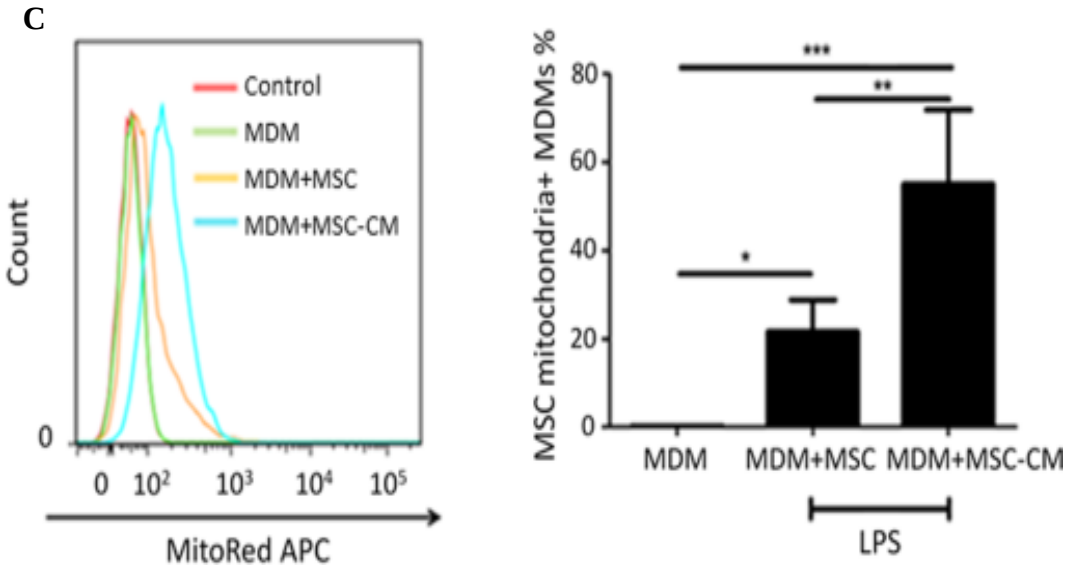



**Fig 5.6 (continued) hMSC-EVs contain mitochondria which are transferred to hMDMs**

MitoTracker Red pre-stained hMSCs or their CM was added to hMDMs with LPS for 24 hours. hMDMs were washed and analysed by flow cytometry to determine the extent of hMSC mitochondrial transfer (C). Error bars represent SD (n=3-5 per group) (One way ANOVA with Bonferroni's post hoc test, *p<0.05, **p<0.01, ***p<0.001).

### 5.9 Mitochondrial transfer via hMSC-EVs mediates their effects on hMDMs

Having confirmed that EV-mediated mitochondrial transfer is taking place, I next determined whether this phenomenon was facilitating the hMSC effects on hMDM function. MitoTracker Red pre-stained hMSCs were co-cultured with hMDMs for 24 hours in the presence of LPS then washed before performing an *E. coli* bioparticle phagocytosis assay. Selective gating was performed to examine the phagocytic indexes of hMDMs which had received hMSC mitochondria and those which had not (determined by positivity for MitoTracker Red). hMDMs which had internalised hMSC mitochondria demonstrated a higher phagocytic index measured by MFI ($p<0.05$) showing an association between mitochondrial transfer and phagocytic enhancement **(Fig 5.7 A). NB: Fig 5.7 A data also appears in Figure 7 of our group's recently published paper (Jackson *et al*, 2016)[116].** To elucidate whether these mitochondria were functionally responsible for the hMSC effects on hMDMs, dysfunctional mitochondria were prepared using rhodamine 6G. Rhodamine 6G has been used previously to irreversibly inhibit mitochondrial function in MSCs, which it achieves through binding to the inner membrane of mitochondria and blocking adenine nucleotide translocase[399, 502]. hMSCs were grown to confluence and treated with rhodamine 6G for 48 hours. The cells were then washed and CM was prepared in LPS. To validate the efficacy of rhodamine 6G in preventing mitochondrial respiration, hMSCs with or without rhodamine 6G pre-treatment were assayed using the Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies). This kit measures oxygen consumption rate (OCR) as an indication of mitochondrial respiration and allows calculation of parameters such as ATP turnover and maximal respiration capacity using oligomycin, FCCP and an antimycin A/rotenone cocktail to modulate mitochondrial activity. hMSCs pre-treated with rhodamine 6G had greatly diminished mitochondrial respiration and ATP turnover with a compensatory switch to glycolysis, evidenced by a dramatic increase in non-mitochondrial respiration **(Fig 5.7 B)**. To assess the effect of rhodamine 6G pre-treatment on hMSC paracrine factor secretion, IL-8 and Ang-1 production was quantified by ELISA after LPS treatment for 24 hours. Rhodamine 6G pre-treated hMSCs produced less Ang-1 (919.3 vs 1403.6 pg/mL) and IL-8 (873.6 vs 1714.9 pg/mL) compared to the untreated controls **(Fig 5.7 C)**. hMSC-CM was added to hMDMs along with LPS for 24 hours and both TNFα production and phagocytosis assays were carried out. Once again, LPS induced a TNFα response

which was suppressed by hMSC-CM (4727.0±812.6 vs 2162.4±860.5 pg/mL) ($p<0.05$). Use of CM from rhodamine 6G pre-treated hMSCs abrogated this effect and hMSCs were no longer able to significantly suppress TNFα secretion (4727.0±812.6 vs 3792.9±1366.4 pg/mL) consistent with the effect observed with anti-CD44 treatment **(Fig 5.7 D)**. Phagocytic capacity was enhanced by hMSC-CM measured by the proportion of phagocytic hMDMs (51.1±13.0 vs 79.8±7.15 %) and MFI (651.8±629.9 vs 3169.2±1137.7) ($p<0.05$). Rhodamine 6G completely reversed the enhancement in phagocytic capacity determined by MFI (651.8±629.9 vs 1284.8±762.6) although interestingly did not have any effect on the proportion of phagocytic hMDMs **(Fig 5.7 E)**. These data suggest that mitochondrial transfer through hMSC-EVs is at least partially responsible for the suppression of inflammation and enhancement of phagocytosis.

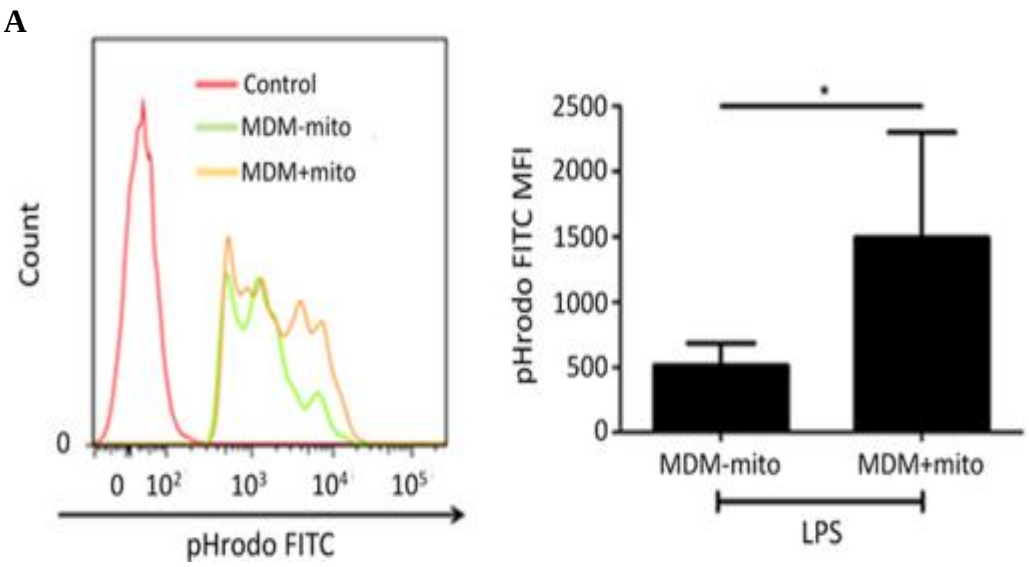



**Fig 5.7 Mitochondrial transfer via hMSC-EVs mediates their effects on hMDMs (continued overleaf)**

hMDMs were treated with hMSC-CM from MitoTracker Red pre-stained hMSCs and *E. coli* bioparticles were added for phagocytosis analysis. hMDMs were gated based on the presence or absence of hMSC mitochondria and phagocytic indexes were compared (A). Error bars represent SD (n=5 each group) (Student's t-test, *$p<0.05$).

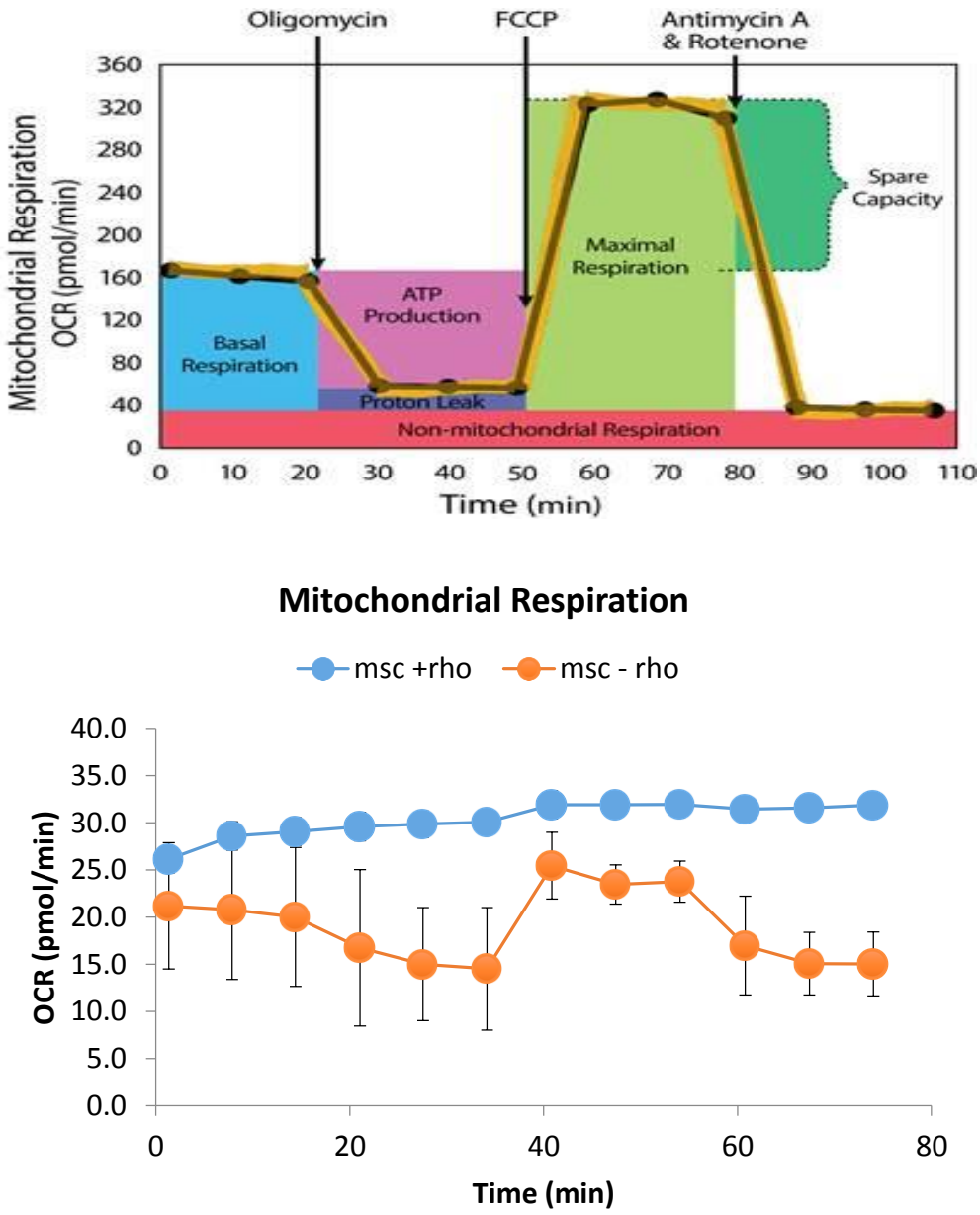



**Fig 5.7 (continued) Mitochondrial transfer via hMSC-EVs mediates their effects on hMDMs (continued overleaf)**

hMSCs were treated with rhodamine 6G for 48 hours, washed and mitochondrial function was assessed using the Mito Stress Test (Bi) (n=1). The measurements produced by the Mito Stress Test and the hMDM oxygen consumption rate (OCR) raw data are presented (pmol/min).

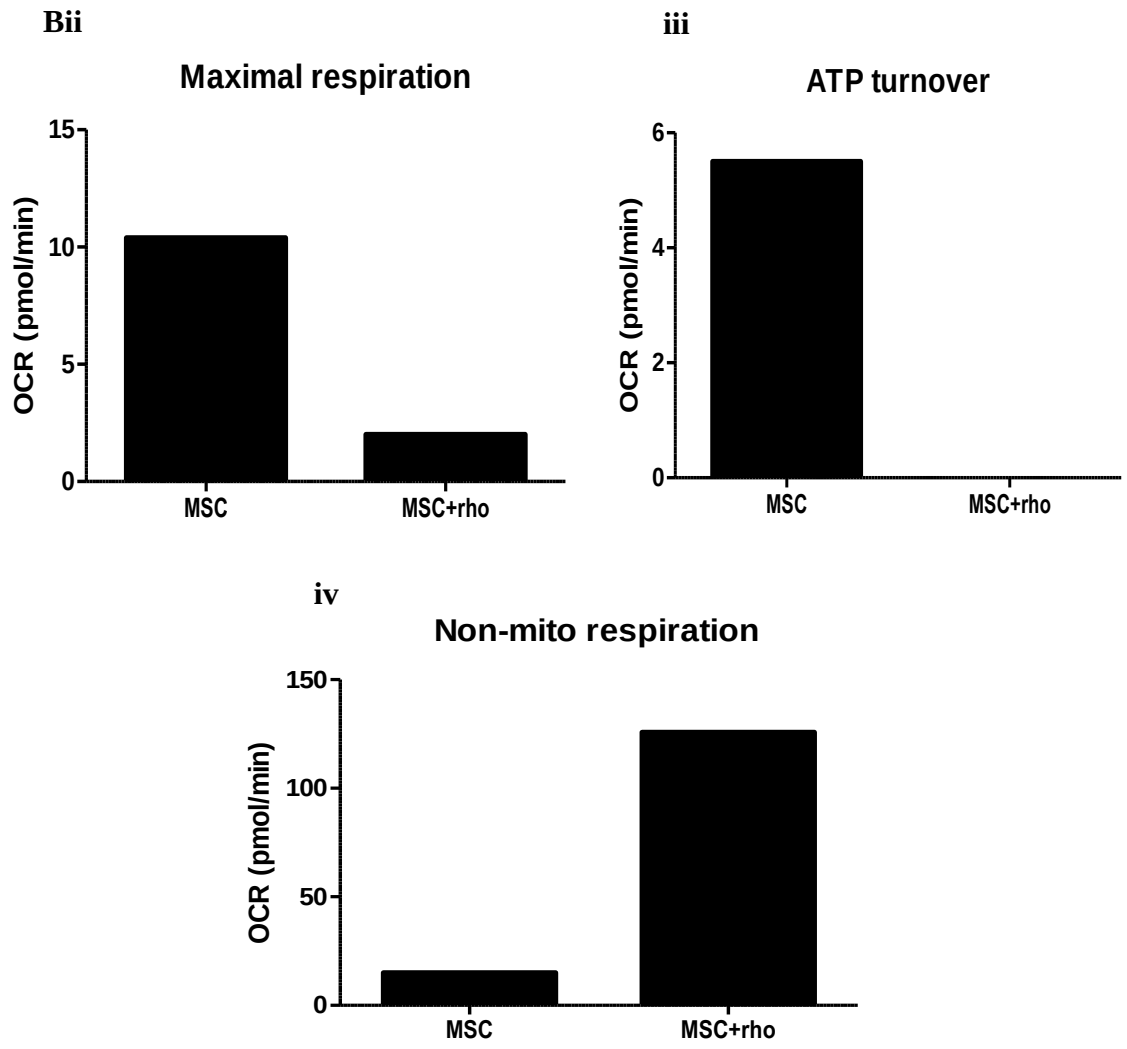



**Fig 5.7 (continued) Mitochondrial transfer via hMSC-EVs mediates their effects on hMDMs (continued overleaf)**

Calculations performed by the Mito Stress Test (n=1); maximal mitochondrial respiration (Bii), mitochondrial ATP turnover (iii) and non-mitochondrial respiration (iv) were quantified to determine the efficacy of rhodamine 6G as a mitochondrial inhibitor.

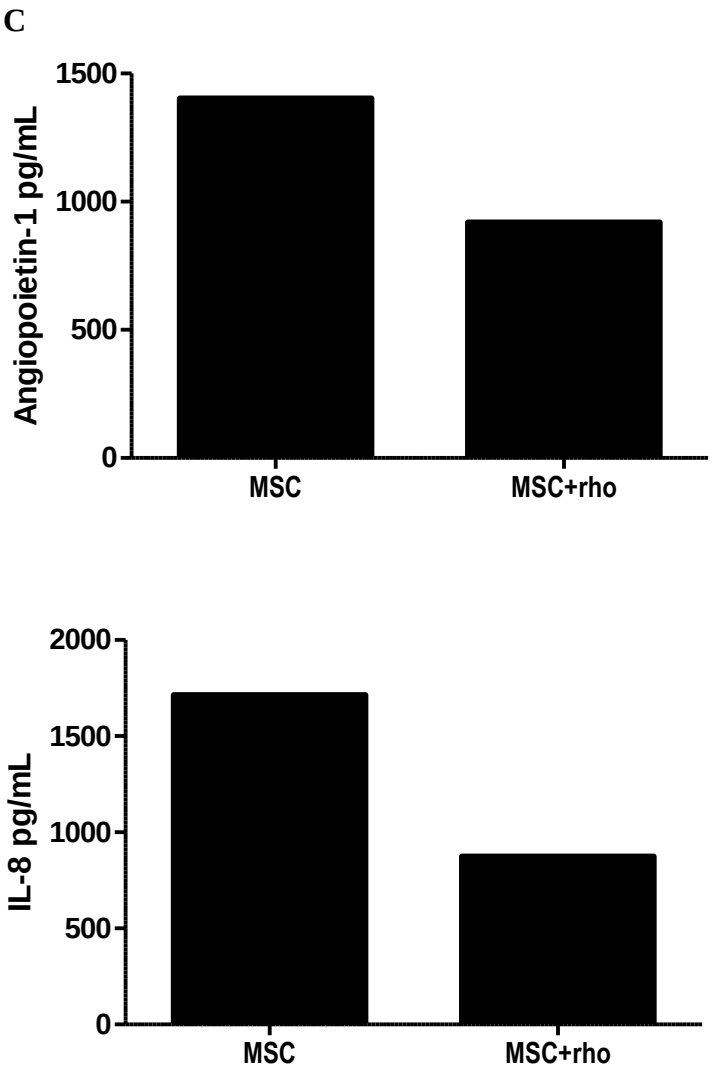



**Fig 5.7 (continued) Mitochondrial transfer via hMSC-EVs mediates their effects on hMDMs (continued overleaf)**

hMSCs pre-treated with rhodamine 6G were stimulated with LPS for 24 hours and their Ang-1 and IL-8 production were measured by ELISA (C) (n=1). Showing average of three technical replicates.

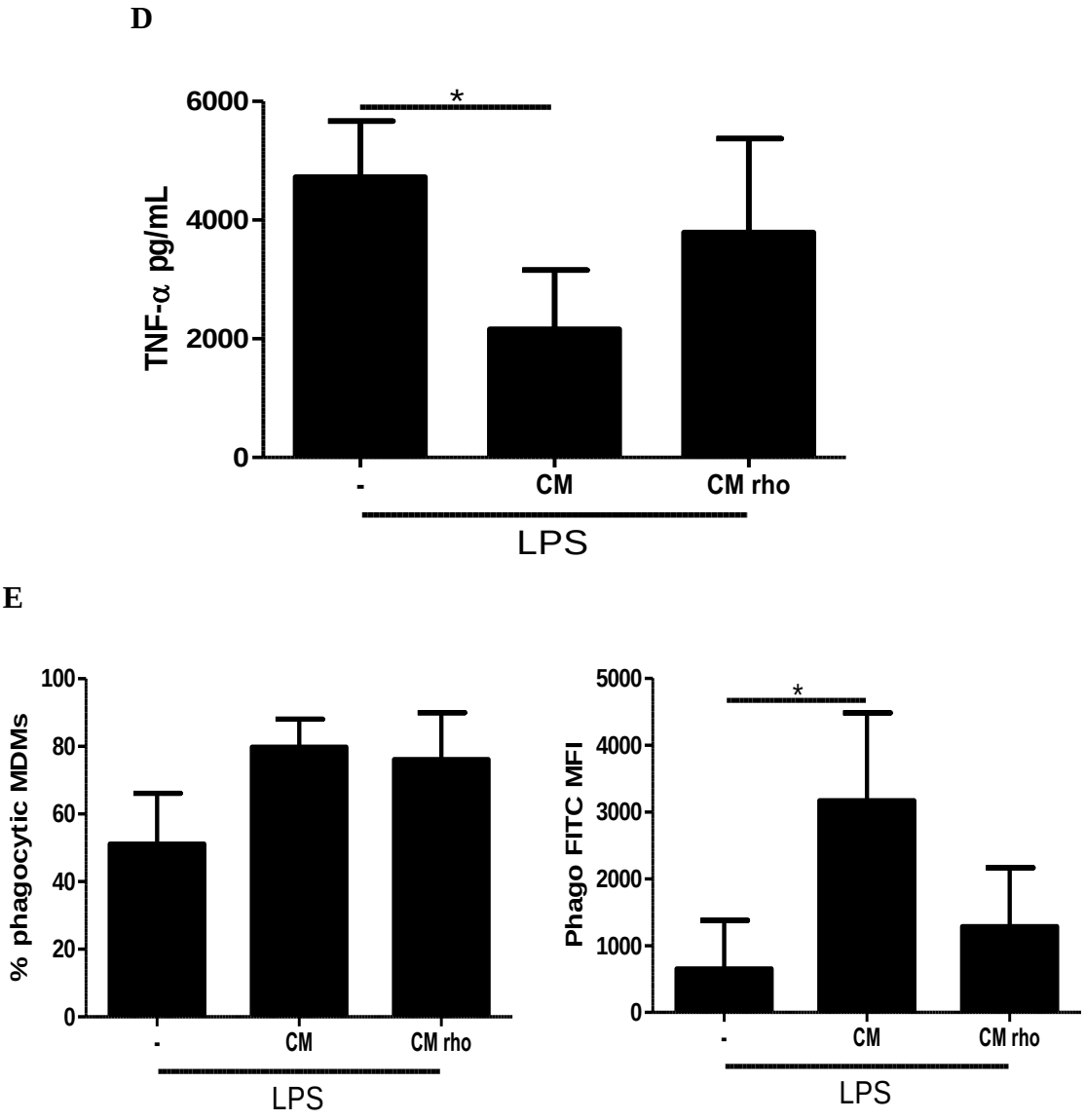



**Fig 5.7 (continued) Mitochondrial transfer via hMSC-EVs mediates their effects on hMDMs**

hMSCs were treated with rhodamine 6G to produce dysfunctional mitochondria and their effects on hMDM TNFα secretion (D) and phagocytosis (E) were assessed. Error bars represent SD (n=4 all groups) (Kruskal Wallis with Dunn's post hoc test, *$p<0.05$).

## 5.10 Mitochondria containing hMSC-EV modulate hMDM function via enhanced oxidative phosphorylation

I hypothesised that the transfer of mitochondria from hMSCs to hMDMs produced the effects on hMDM function by modulating their metabolic activity through promoting oxidative phosphorylation. As proof of concept, the ATP synthase inhibitor oligomycin was used[503]. hMDMs were stimulated with LPS and oligomycin was added simultaneously to determine whether blocking oxidative phosphorylation influenced TNFα production and phagocytosis. DMSO was added as a vehicle control. LPS-induced TNFα production by hMDMs was amplified with the addition of oligomycin (3788.6±2199.5 vs 14385.0± 3290.9 pg/mL) ($p<0.05$). Oligomycin also prevented hMSC-CM suppression of TNFα production (1108.8±914.6 vs 9415.9±6590.9 pg/mL) ($p<0.05$) and in fact increased it beyond the levels produced by LPS-stimulated hMDMs, albeit non-significantly (3788.6±2199.502 vs 9415.9±6590.8 pg/mL) **(Fig 5.8 A)**. Oligomycin produced a trend towards decreasing phagocytic capacity of hMDMs stimulated with LPS by both percentage phagocytic hMDMs (54.3±15.0 vs 33.6±16.7 %) and MFI (1359.9±1916.8 vs 349.6). Oligomycin significantly diminished phagocytic activity of hMDMs in the presence of hMSC-CM by both percentage (80.1±5.6 vs 31.9±21.7 %) ($p<0.001$) and MFI (4261.6±2493.2 vs 378.5±439.6) ($p<0.01$) **(Fig 5.8 B)**. Thus, the effect of hMSC-CM was dependent on their enhancement of oxidative phosphorylation. Next, in order to determine whether hMSC-CM enhanced mitochondrial respiration directly, I used the Mito Stress Test once again. hMDMs were treated with LPS, as well as hMSC-CM from untreated or rhodamine 6G-treated hMSCs and the Mito Stress Test was carried out. Only two donors were obtained to perform this experiment and so there was no statistical analysis performed. The raw data plots for OCR are shown along with the calculations used to determine mitochondrial activity parameters **(Fig 5.8 C)**. Basal mitochondrial respiration rate was enhanced by hMSC-CM in the presence of LPS (8.3±1.1 vs 13.62±0.3 pmol/min). Maximal mitochondrial respiration rate was also increased (40.7±4.6 vs 52.3±5.7 pmol/min) **(Fig 5.8 D)**. Additionally, ATP turnover was trending upwards with addition of hMSC-CM (6.9±2.1 vs 9.2±0.2 pmol/min) **(Fig 5.8 E)**. The pre-treatment of hMSCs with rhodamine 6G to generate dysfunctional mitochondria, abrogated the apparent increase in basal respiration rate (8.3±1.1 vs 10.54±1.23 pmol/min), maximal respiration rate (40.7±4.6 vs 43.0±1.1 pmol/min) and

ATP turnover (6.9±2.1 vs 7.4±0.8 pmol/min) that was present with untreated hMSC-CM **(Fig 5.8 D and E)**.

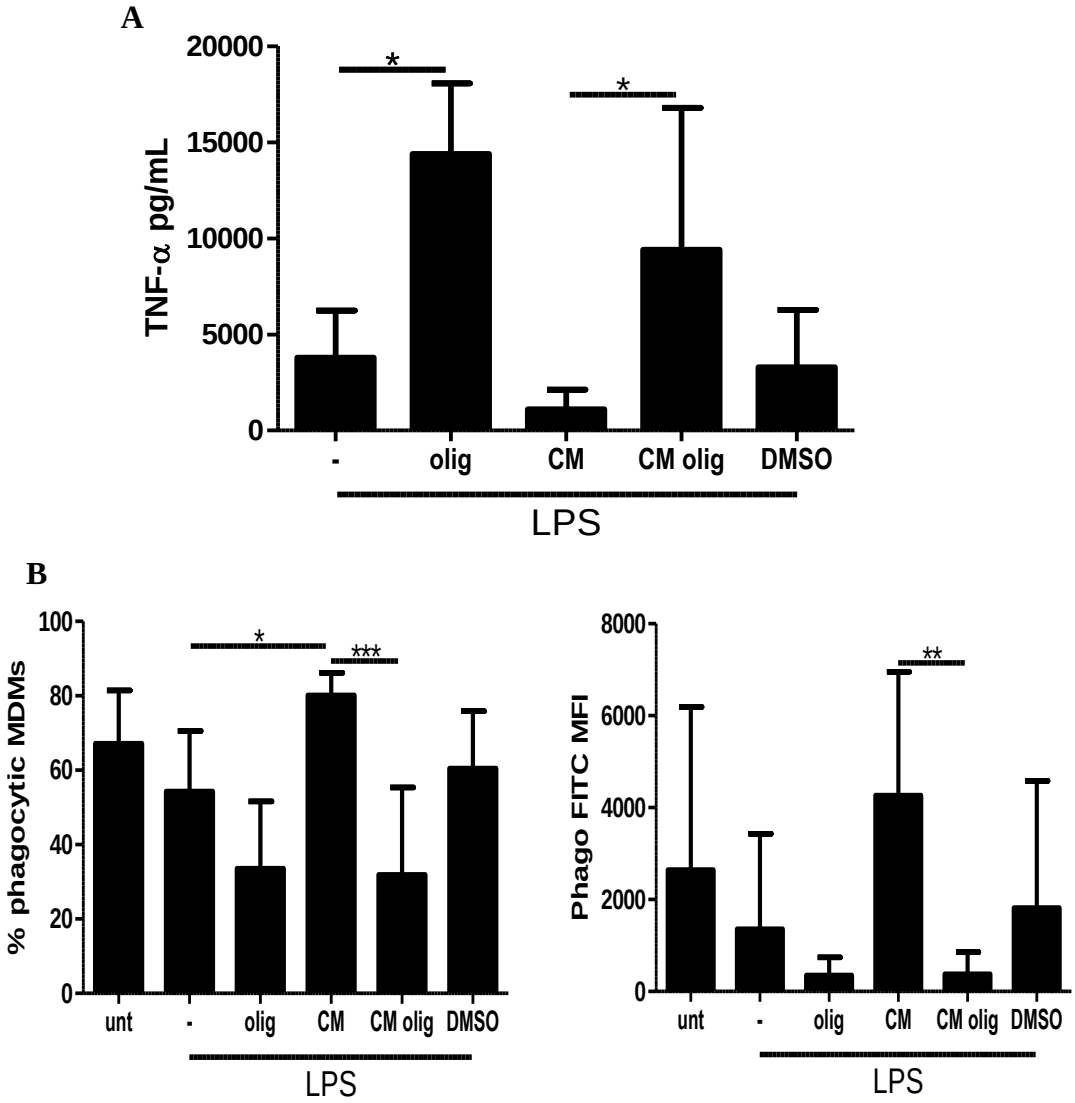



**Fig 5.8 The effect of hMSCs on hMDM function is dependent on oxidative phosphorylation (continued overleaf)**

hMDMs were stimulated with LPS with or without oligomycin as well as hMSC-CM. Supernatants were lifted after 24 hours for TNFα measurement (A) (n=4 for DMSO group, n=5 for remaining groups). *E. coli* bioparticles were added to assess phagocytosis, presented as a percentage and by MFI (B) (n=7 all groups). Error bars represent SD (Kruskal Wallis with Dunn's post hoc analysis, *p<0.05, **p<0.01, ***p<0.001).

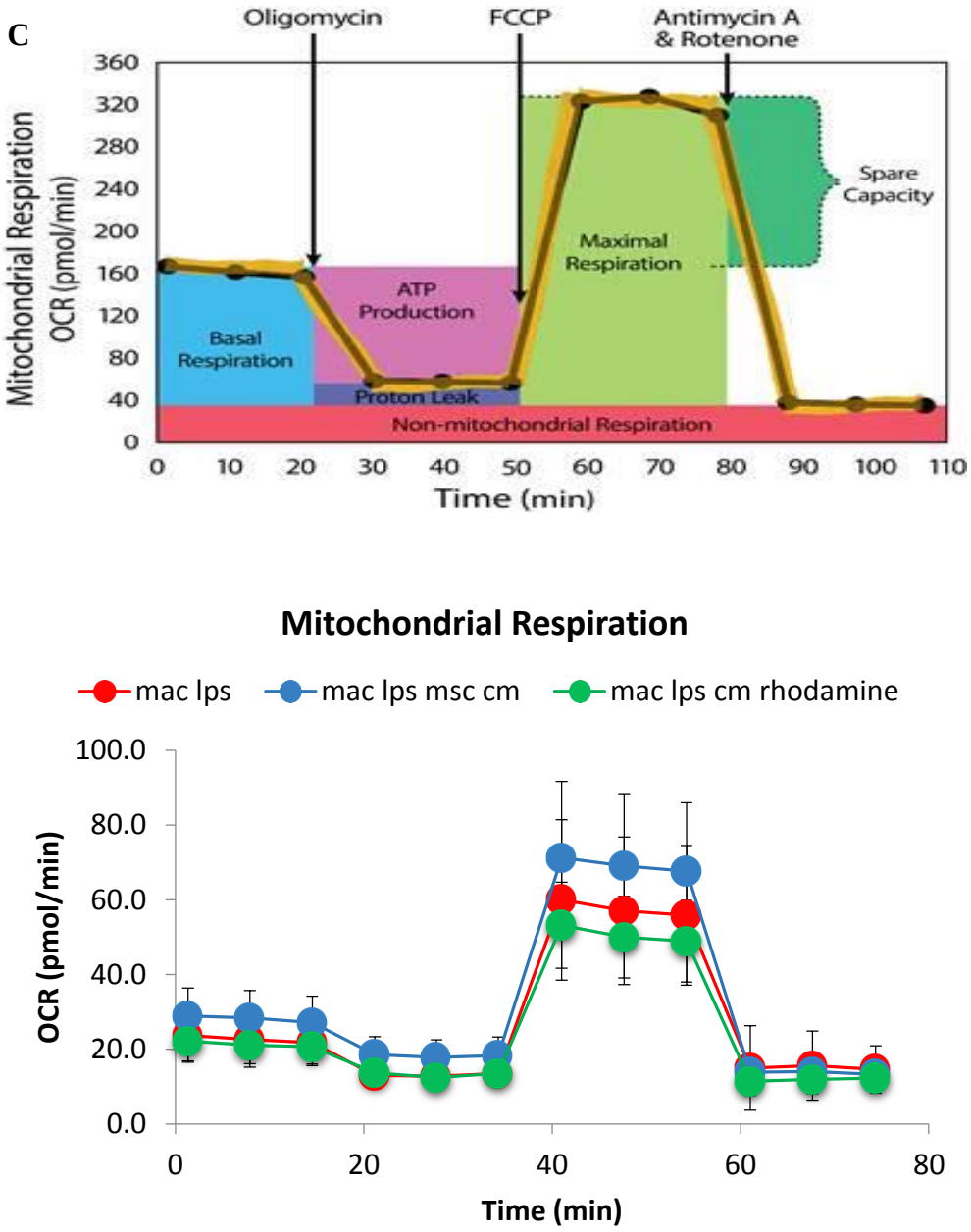



**Fig 5.8 (continued) The effect of hMSCs on hMDM function is associated with enhanced oxidative phosphorylation (continued overleaf)**

hMDMs were stimulated with LPS and CM from hMSCs with or without rhodamine 6G pre-treatment and mitochondrial respiration was evaluated. The measurements produced by the Mito Stress Test and the hMDM OCR raw data is presented (pmol/min) (C). Error bars represent SD (n=2). Results from one experiment are shown.

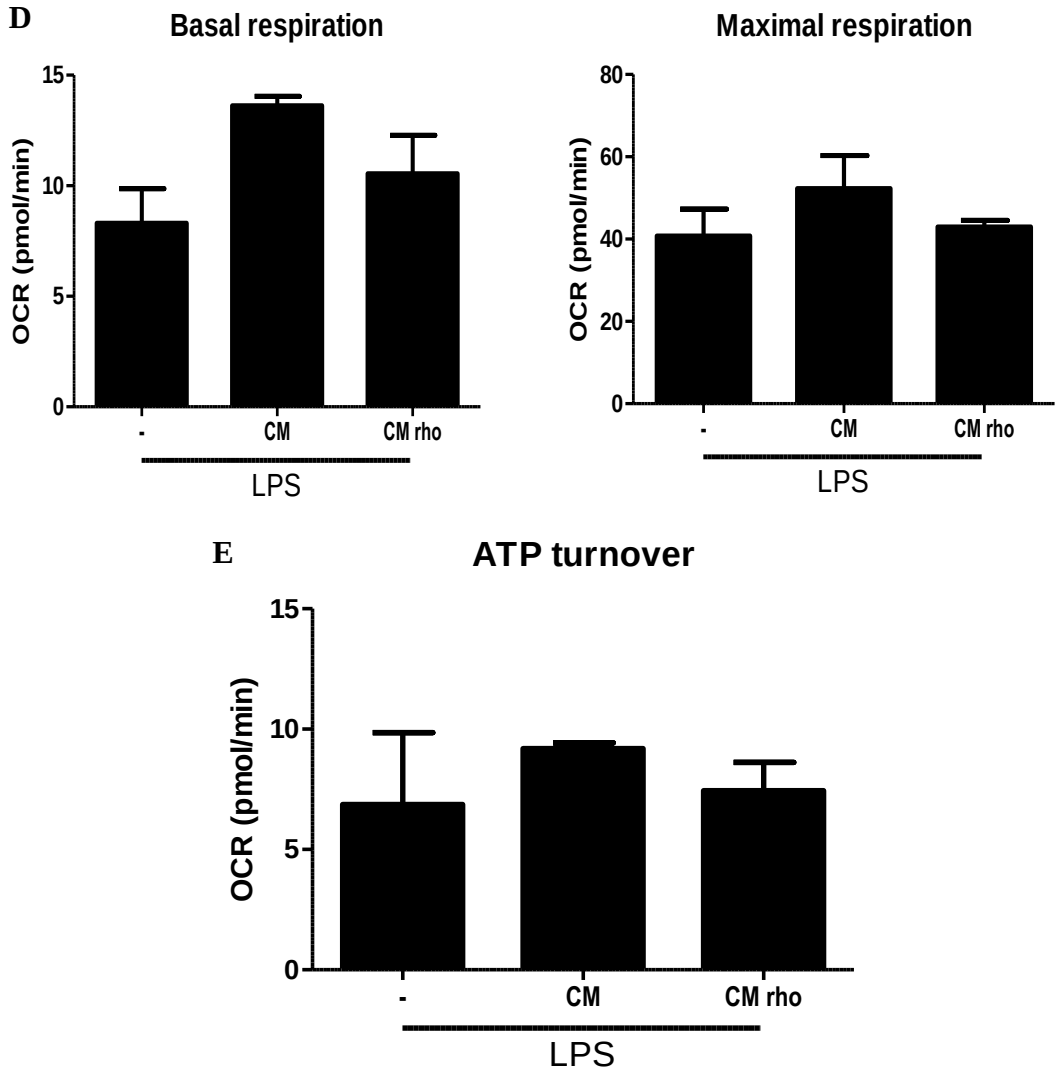



**Fig 5.8 (continued) The effect of hMSCs on hMDM function is associated with enhanced oxidative phosphorylation**

Calculations of mitochondrial function were produced using the Mito Stress Test. Basal and maximal mitochondrial respiration rates (D), as well as ATP turnover rate (E) are presented by OCR (pmol/min). Error bars represent SD (n=2).

## 5.11 Discussion

After unsuccessful attempts to identify soluble mediators responsible for the paracrine effects of hMSCs, EVs were hypothesised to be responsible for the macrophage modulating effects of hMSC-CM. Monsel *et al* have already shown that MSC-EVs were protective in the *in vivo* model of *E. coli* pneumonia and demonstrated that CD44 expression on the EV surface was required for uptake by recipient cells[171]. The CD44 family are transmembrane glycoproteins known for their roles in ECM and cellular adhesion, cell migration, immune cell activation and limb development[504-508]. CD44 has also been implicated in MSC migration through the ECM and in trafficking to sites of injury after intravenous injection[509, 510]. Before testing the potential role of hMSC-EVs in the hMSC effect, the EVs were first characterised by flow cytometry. hMSC-CM was analysed alongside latex beads of known size to provide an indication of the size of these EVs. FSC vs SSC plots demonstrate that these EVs are smaller than the latex beads (4μm) which is consistent with the literature showing that the larger microvesicle component of EVs can range from 0.4 to 1 μm in diameter. It was important to rule out potential contaminating apoptotic and necrotic bodies in these preparations. This was achieved by staining with annexin V, which has a high affinity for phosphatidylserine; this phospholipid is only present on the exterior face of the plasma membrane during apoptosis[511]. Propidium iodide staining was used to rule out necrotic cell debris. Additionally, using flow cytometry allows for protein aggregates, which commonly contaminate ultracentrifuged EV isolations, to be ruled out using a cell membrane stain[512]. The limitation of this method is that conventional flow cytometers are not sensitive enough to detect smaller EVs (exosomes) and so it is likely that these were not accounted for. Dedicated flow cytometers with increased sensitivity are available for EV flow cytometry but conventional cytometers may be used and can reportedly detect vesicles ranging from 270-600nm in diameter[513-515]. Phinney *et al* used electron microscopy to visualise MSC-EVs and observed exosomes below 100nm in diameter, mitochondria-containing EVs and multivesicular bodies with diameters beyond 400nm[291]. Flow cytometry for the present study demonstrated that nearly all of the EVs that the cytometer could detect were positive for CD44 and 25.2% of these EVs contained mitochondria.

The importance of CD44-expressing EVs was assessed by antibody-based neutralisation experiments. It was necessary to add anti-CD44 antibody to hMDMs without hMSC-CM. The purpose of the antibody is to bind the CD44 present on the EV membranes that reside within the hMSC-CM so that it cannot be taken up by the hMDMs. It is known that macrophages also express CD44 which plays a number of functional roles; CD44 is an important mediator in the resolution of inflammation through facilitating apoptotic neutrophil clearance and contributing to the activation of TGFβ[516, 517]; CD44 can even suppress TLR2 signalling in macrophages leading to reduced NFκB activation[518]. Furthermore, the hyaluronan receptor (CD44) is integral to the ingestion of *M. tuberculosis* by macrophages[372]. As expected, hMSC-CM resulted in the suppression of TNFα secretion and the enhancement in phagocytosis. Notably, the enhancement in phagocytosis with CM is much more pronounced than occured with hMSC co-culture in **Chapter 3 (Fig 3.4)**, specifically determined by MFI (phagocytic index). One explanation for this is that using CM produced for 24 hours by hMSCs will contain paracrine factors including EVs which are presented to the hMDMs immediately. hMSCs in non-contact co-culture are added at the time of LPS stimulation and have to then produce these factors anew which will take 24 hours to reach the levels present in the CM. Therefore, hMDMs treated with hMSC-CM are being influenced with these regulatory factors at this higher concentration for what is effectively a longer time. Moreover, the increased phagocytic augmentation in CM-treated hMDMs may be explained by the nature of the co-culture system used. hMSCs were cultured on transwell inserts containing 0.4μm pores, these pores are too small for the largest EVs to move across and reach the hMDMs. That means that the contents of these larger EVs will not be accounted for in the paracrine effect on hMDMs in the non-contact co-culture experiments. Naturally isotype control antibody was also included and was added to hMDMs with or without hMSC-CM. This is of importance to rule out non-specific effects of antibody binding but especially important in this experiment given the body of literature suggesting the influential role of immune complexes on macrophage phenotype and function. For example, interactions with the FcγR of the macrophage result in an M2 phenotypic shift, evidenced by their induction of Th2 lymphocyte responses[519].

Treatment of hMDMs with isotype antibody or anti-CD44 alone trended towards suppressing TNFα production. hMSC-CM significantly reduced TNFα secretion by hMDMs and anti-CD44 treatment partially abrogated this effect indicating that CD44-expressing EVs are at least partially responsible for the hMSC-CM effect on TNFα secretion. A number of explanations could be proposed for the absence of significance here. Firstly, the trend towards inhibiting TNFα secretion with anti-CD44 treatment of hMDMs without hMSC-CM suggests that the potential reversal in TNFα suppression by blocking EV uptake was masked by the additional suppression afforded by anti-CD44 interactions with hMDMs directly. Another explanation is that hMSCs produce multiple immunoregulatory paracrine factors; PGE2, IDO and IL-1ra to name a few[520]. It is unlikely that blocking just one such factor would completely reverse the anti-inflammatory effect of hMSCs on hMDMs given that so many other elements of the MSC regulatory repertoire are still active. Furthermore, it is likely that flow cytometry could not characterise the smaller exosomal component of the EVs which may have lacked CD44 expression and still have been capable of exerting some anti-inflammatory effects. Anti-CD44 did however significantly reverse the phagocytic enhancement seen with hMSC-CM. Isotype and anti-CD44 antibody treatment of hMDMs without hMSC-CM had no effect on phagocytosis. Reversal of phagocytic enhancement by blocking CD44 in hMSC-CM suggests that CD44-expressing EVs found in this CM are mediating the effect.

All of the results thus far have been based on *in vitro* experiments. To demonstrate the importance of EV transfer to macrophages in the protective effect of hMSCs in lung injury, an adoptive transfer experiment was conducted where murine AMs were treated *ex vivo* with hMSC-EVs and then administered to *E. coli* endotoxin-injured mice. Results showed that treatment of LPS-injured mice with EV-treated AMs mitigated lung injury as determined by inflammatory cellular infiltrates and the amount of protein present in the BALF. Importantly, AMs cultured *ex vivo* without EV treatment were unable to reduce the extent of lung injury. Inflammatory cellular infiltration is a key feature of ARDS; neutrophils and monocytes are recruited from the periphery and damage the alveolar epithelium through the release of pro-apoptotic factors[20, 22, 23, 273, 521]. Total cell counts in the BALF were measured and the various cell types present are shown by brightfield microscopy. In non-injured, PBS-treated

mice there are primarily myeloid type cells and a few epithelial cells present. With LPS injury there is a large influx of neutrophils, macrophages and erythrocytes into the BALF. EV-treated-AM-treated mice demonstrated a lower total cell count and absolute neutrophil cell count compared to the LPS group indicating reduced inflammation. Decrease in absolute neutrophil counts is of special importance since neutrophil infiltration is a hallmark of ARDS[20, 522, 523]. Myeloid cell numbers, in this case, would have been confounded by the administration of *ex vivo*-cultured AMs. Reduced inflammation was associated with decreased protein in the BALF, indicating greater alveolar epithelial-endothelial integrity in these mice. This demonstrates that EVs represent an essential component of the hMSC secretory profile capable of recapitulating the beneficial effects of hMSCs through reduction of inflammation and lung injury. This finding also supports the concept that AMs are key cellular mediators of the hMSC-EV protective effect in lung injury. Indeed, a number of groups are investigating the potential of macrophage adoptive transfer as a therapy. Andreesen *et al* tested the safety and efficacy of peripheral blood MDMs, primed with IFNγ given IV and IP to cancer patients. They observed some side effects and disappearance of malignancies in a small proportion of patients[524]. Leung *et al* demonstrated the potential of adoptive transfer of macrophages in an experimental colitis model. Treatment with alternatively activated bone marrow-derived macrophages resulted in reduced histopathological severity[525]. The crucial importance of AMs in mediating the hMSC effect in lung injury is consistent with our group's previously published work investigating the effect of macrophage depletion in *E. coli*-induced pneumonia. Treatment with hMSCs no longer resulted in reduced bacterial load, neutrophilia or protein in the BALF after clodronate depletion of AMs[116]. The AM-dependent nature of the hMSC effect further highlights the importance of AM in driving the inflammatory response in lung injury and identifies AMs as an ideal target for therapy.

The mild model of lung injury using a relatively low dose of endotoxin (20μg per mouse) was chosen to maximise the chances for hMSC-EV-educated AMs to achieve therapeutic effect. The results should be considered as a proof of concept that macrophages are key cellular mediators of the MSC effect in ARDS, the next step would be to extend these studies to a more relevant model, such as live *E. coli* or sepsis-induced lung injury. The major technical obstacles in performing these

experiments were isolating substantial numbers of AMs for *ex vivo* culture and isolating sufficient yields of EVs from hMSC-CM to treat them. For that reason it was decided to treat each mouse with $2.5x10^5$ AMs. The results so far suggest that hMSC-EVs influence macrophage function and that these educated macrophages are protective in lung injury. The next step was to determine the contents of these EVs that are specifically facilitating these effects. Given that MSC-EVs are known to contain miRNAs and that these miRNAs have been shown to downregulate TLR signalling, it was hypothesised that miRNAs within hMSC-EVs were responsible for inhibiting TNFα secretion[291]. Reviewing the literature, three potential candidates were identified; miR-125a, miR-125b and miR-223 which are known to modulate macrophage function [291, 347, 399, 497, 498]. These candidates were all detected within hMSCs and their EVs. miR-125b was the most abundant and so was investigated further. Inhibitor validation tests demonstrated some non-specific effects; anti-miR-125b inhibitor also suppressed miR-125a and vice versa. Therefore subsequent inhibition experiments with anti-miR-125b were effectively testing whether miR-125a or miR-125b played a role in the hMSC effect. The results suggest that this is not the case. It cannot be ruled out that miR-125a and miR-125b may have had divergent and opposite influences on these hMDMs effectively masking the effects of inhibiting one or the other. This seems improbable however, based on reports of their functions in macrophages and the nature of their homology. Phinney *et al* show that MSC-EV miRNAs do play a role in modulating TLR signalling but the specific miRNA/s responsible remain elusive[291].

Given that mitochondria are contained in CD44-expressing vesicles and our group's findings that contact-dependent mitochondrial transfer augments macrophage phagocytic capacity[116], it was hypothesised that EV-dependent mitochondrial transfer was responsible for the hMSC effect on macrophage modulation. So far, these results have shown that hMSC-EVs contain mitochondria but their effective transfer to hMDMs was yet to be confirmed. The adherence of hMSC-EVs containing mitochondria to hMDMs was observed by fluorescence microscopy. Internalisation and integration into the hMDM mitochondrial network is apparent through co-localisation of fluorescent staining. Interestingly, the co-localisation is distinctly localised rather than assuming a more diffuse distribution. Our group's previous paper

studying non-contact co-culture shows much more diffuse mitochondrial transfer[116]. Tunnelling nanotubules likely represent a more efficient means of donating mitochondria allowing greater numbers to integrate into the macrophage network. Phinney *et al* were investigating mitophagy and EV-mediated transport and also observed more substantial mitochondrial transfer than my results show[291]. Importantly however those cultures were also with MSC-macrophage contact and so the rate of EV-mediated transfer would be much higher. Mitochondrial transfer to hMDMs via EVs was further supported by flow cytometry; hMSCs cultured on transwell inserts donated mitochondria to 21.7% of hMDMs which was increased to 55.1% using hMSC-CM. Gating for hMDMs which had received hMSC mitochondria and those which had not demonstrated an association between mitochondrial transfer and phagocytic capacity but not causation.

To test whether hMSC mitochondria were functionally responsible, dysfunctional mitochondria were generated using rhodamine 6G which irreversibly inhibits adenine nucleotide translocase function[502]. CM from hMSCs pre-treated with rhodamine 6G was unable to reduce TNFα secretion in hMDMs stimulated with LPS, nor was it able to promote phagocytic activity measured by MFI. This loss-of-function experiment suggests that functional mitochondria transfer to hMDMs is required for both TNFα suppression and phagocytic enhancement by hMSCs. Regarding phagocytosis, this causation correlates with the association already seen between mitochondrial transfer and phagocytic activity. One concern about this loss-of-function experiment is that producing dysfunctional mitochondria and inducing glycolytic metabolism could somehow affect other hMSC functions such as paracrine factor production. To address this, rhodamine 6G pre-treated hMSCs were treated with LPS and their production of Ang-1 and IL-8 was compared to that of untreated hMSCs. There was a downregulation of production but importantly the levels after rhodamine 6G treatment were still substantial. Rhodamine 6G pre-treatment does not drastically ablate paracrine factor production but it did completely inhibit mitochondrial function and so the effects observed can be attributed to the absence of functional mitochondria. It is also worth considering the results from the oligomycin experiments when considering the reliability of the rhodamine 6G experiment. hMDMs that were given hMSC-CM produced less TNFα and adding oligomycin not only prevented this but

actually increased production further than the LPS alone group (non-significantly). In the CM and oligomycin-treated group hMDMs would have been exposed to hMSC mitochondria and all paracrine factors that they secrete; in spite of this, TNFα was no longer reduced. The same was true of the enhancement in phagocytosis; oligomycin completely reversed the phagocytosis enhancing effect of hMSC-CM. Oligomycin could only possibly block the function of mitochondria, certainly of either hMSC or hMDM origin, but importantly other paracrine factors could interact with the hMDMs unabated. This supports the hypothesis that it is the transfer of functional mitochondria and not only soluble mediators that are responsible for the hMSC effects. Secondly, it highlights the importance of mitochondrial respiration in modulating macrophage functions. Promotion of mitochondrial respiration using oxidative phosphorylation seems to dampen inflammatory responses, whereas LPS-activated M1 macrophages will preferentially employ glycolysis[240, 526]. Oligomycin forces hMDMs down the glycolytic pathway amplifying the metabolic effects of LPS alone, promoting a more pronounced M1 macrophage phenotype. This corresponds to increased pro-inflammatory cytokine secretion and even further reduced phagocytosis which is observed in response to LPS alone in the current study. In order to confirm the hypothesis that EV-mediated mitochondrial transfer is responsible for hMSC effects *in vivo*, rhodamine pre-treated hMSC-derived EVs could be used in an adoptive transfer experiment. A limitation of this *in vivo* model however is that without using live bacteria, the antimicrobial effects of EV-treated AMs cannot be assessed.

So far in this chapter, these results suggest that mitochondria-containing EVs produced by hMSCs are actively taken up by hMDMs and that this transfer is entirely responsible for their enhancement in phagocytosis but only partially responsible for the suppression of TNFα production. In order to investigate this mechanism in more depth, the extent of mitochondrial respiration in hMDMs treated with LPS, LPS + hMSC-CM and LPS + rhodamine 6G pre-treated-hMSC-CM was assessed. It was hypothesised that the transfer of hMSC mitochondria would increase the rate of oxidative phosphorylation and mitochondrial ATP production which could explain the dampened inflammatory response and enhanced phagocytosis of hMDMs. These experiments were performed on two donor's hMDMs and so the results are inconclusive. The trends were in agreement with the hypothesis; an increase in basal

and maximal mitochondrial respiration as well as ATP turnover was observed with hMSC-CM treatment. This finding is in keeping with previous reports showing that mitochondrial transfer from MSCs results in enhanced bioenergetics of macrophages and alveolar epithelial cells[116, 291, 318]. Interestingly, when hMSCs were pre-treated with rhodamine 6G, their CM no longer improved hMDM bioenergetics suggesting that it was the hMSC-derived mitochondria responsible for this effect. The loss of hMSC effect with rhodamine 6G pre-treatment or with addition of oligomycin, suggests that hMSCs are able to influence hMDM phenotype and function by donating mitochondria and promoting oxidative phosphorylation. This study has not however tested whether there is any influence of hMSCs on the extent of hMDM glycolysis. It would be interesting to determine if there is a reciprocal downregulation of glycolysis and if that plays a role in mediating hMSC modulation of hMDM phenotype.

## 5.12 Summary and conclusions

The key findings from these studies are:

- CD44-expressing EVs are primarily responsible for the paracrine effects of hMSCs on hMDM modulation
- hMSC-EVs are essential for AM modulation *in vivo*
- hMSCs modulate hMDM TNFα secretion and enhance their phagocytic capacity through the release of mitochondria-containing EVs
- hMSC modulation of hMDM function depends on oxidative phosphorylation
- Preliminary data suggests that the transfer of mitochondria to hMDMs may promote oxidative phosphorylation, producing the phenotypic shift attributable to hMSCs.

Collectively these studies reveal a new mechanism of hMSC paracrine effect, highlights the role of AMs as cellular mediators of the MSC effect in ARDS *in vivo* and provide evidence that promotes further development of MSC-derived EVs and MSC-educated macrophages as potential therapeutic approaches for the treatment of ARDS.

# Chapter 6
# Final conclusions and future directions

## 6.1 Final conclusions

ARDS is a highly prevalent clinical disorder in ICUs worldwide which is associated with unacceptably high mortality rates and significant morbidity in survivors[5, 527-529]. Due to improvements in quality of care, primarily through the use of low-volume mechanical ventilation, mortality rates have declined modestly with time[4, 530]. As yet however, there is no pharmacologically effective treatment available. MSCs have piqued the interest of basic scientists and clinicians as a potential therapy for ARDS with a growing body of literature supporting this notion through pre-clinical studies[77, 81, 93, 318]. Indeed, MSCs have proved safe and feasible for their administration to ARDS patients in phase I clinical trials[130, 131] and a larger phase II clinical trial is underway (ClinicalTrials.gov identifier: NCT02097641). The mechanisms by which MSCs exert their therapeutic effects in models of lung injury are not fully elucidated. A fuller understanding of their mechanisms will allow the rational development of MSC-based therapy through generation of disease specific MSC potency assays. Moreover it may identify novel biomarkers to test MSC efficacy in clinical samples. For example, patient BALF may be processed by flow cytometry to assess macrophage expression of CD206 which could serve as a biomarker for the efficacy of MSC treatment. Potency assays will be crucial for batch testing of MSCs to determine which preparations will be consistent and effective clinical-grade cell therapies. Furthermore, discovering new mechanisms of MSC effect may highlight new therapeutic strategies or targets for treatment of patients with ARDS.

MSCs are renowned for their immunomodulatory capacity, including their interactions with macrophages[289, 404, 468]. The AM is the resident innate immune cell of the distal lung which is implicated in the initiation and resolution of inflammation in lung injury[273, 390]. Modulation of AM phenotype and function, therefore, may prove beneficial in treatment of ARDS and could represent one mechanism by which MSCs have proven successful in preclinical models of lung injury. The major aims of this project were to investigate the effects of hMSCs on human macrophage phenotype and function in *in vitro* models of ARDS and to elucidate the mechanism(s) of these effects. hMSCs were shown to induce an unconventional M2-like hMDM phenotype in *E. coli* LPS or ARDS BALF-induced inflammation. This phenotype was consistent across both *in vitro* models and was defined by a dampened inflammatory cytokine

profile, increased expression of the CD206 M2 macrophage marker and enhanced phagocytic capacity. This macrophage phenotype could feasibly reduce inflammation and injury in ARDS whilst maintaining bacterial clearance in infected patients. The macrophage phenotype described in this study corroborates the findings of other groups studying the influence of MSCs on macrophage phenotype. Deng *et al* reported induction of CD206 expression in mouse macrophages with increased phagocytic capacity in systemic lupus erythematosus[439]. Kim and Hematti similarly showed increased expression of CD206, reduced TNFα production and accelerated phagocytosis in hMDMs cultured with hMSCs[287]. MSC promotion of alternatively activated macrophages is also evident in *in vivo* models; Nemeth *et al* showed increased IL-10 production by macrophages which was necessary for the beneficial effects observed in MSC-treated septic mice[85]; AMs treated with MSCs in a model of asthma also adopt and M2 macrophage phenotype resulting in reduced airway hyperresponsiveness[531]. Where these studies differ from the current study is in the effects seen on anti-inflammatory cytokine production. Many of these groups have reported increased production of IL-10 in the presence of MSCs. This discrepancy could be explained by differences in the source of macrophages or their culture conditions. Variations in differentiation protocols and inflammatory stimuli can influence macrophage phenotype[411, 417]. Moreover, the methods used in these studies differ from those employed here; Kim and Hematti tested intracellular IL-10 expression by flow cytometry after treatment with 1μg/mL of LPS; Nemeth *et al* studied mouse MSCs and mouse macrophages. This is not the first study to report a lack of IL-10 production by human macrophages expressing an otherwise M2-like phenotype; Vogel *et al* reported that IL-10 production in human macrophages was negligibly low after a variety of M1 and M2 polarising stimuli[411]. To our knowledge, this is the first study to test the influence of hMSCs on human macrophage polarisation in the presence of ARDS patient BALF. These results suggest that hMSCs are capable of inducing the same M2-like phenotype in ARDS BALF as they are with LPS treatment. This may provide valuable insight into the interactions that would occur between administered hMSCs and the AMs of ARDS patients and may represent a mechanism of their effect.

The second aim of this project was to determine the mechanism(s) of hMSC effects on hMDM phenotype and function. EVs produced by hMSCs have previously been shown to mediate many of their effects in lung injury and CD44 expression on the surface of these EVs was important for their uptake[171, 317]. In the current study, anti-CD44 antibody pre-treatment of hMSC-CM prevented their enhancement of hMDM phagocytosis and partially blocked the suppression of TNFα production. These results suggest that hMSC CD44-expressing EVs are important for hMSC modulation of hMDM function. Similarly, Monsel *et al* showed that hMSC-EVs enhance human monocyte phagocytosis and mitigate the TNFα response to *E. coli* in vitro[171]. The importance of hMSC-EV modulation of AMs was confirmed in an endotoxin-induced lung injury mouse model where adoptive transfer of hMSC-EV-treated AMs conferred protection. EV-treated AMs were able to reduce inflammatory cell recruitment and rescue alveolar epithelial barrier permeability. These data highlight the AM as an important cellular mediator of the hMSC therapeutic effect in lung injury. These results consolidate findings by our group previously, where depletion of AMs using clodronate-containing lysosomes prevented the beneficial effects of hMSCs in *E. coli*-induced lung injury[116]. Nemeth *et al* also showed that depleting macrophages systemically abrogated the MSC effect in a murine sepsis model[85]. There are limitations to the lung injury model chosen here. This is a model of mild endotoxin-induced lung injury which is of limited relevance to ARDS. The purpose of this experiment was not to test the capacity of hMSC-EV-treated AMs as a treatment for lung injury; it was to show proof-of-concept that AMs help facilitate the protective effects of hMSCs in lung injury. Some groups have investigated the feasibility and efficacy of macrophage adoptive transfer as a treatment modality in cancer and renal fibrosis *in vivo*[524, 532]. Leung *et al* were able to reduce the severity of experimental colitis with the adoptive transfer of bone marrow-derived alternatively activated macrophages[525]. Careau and Bissonnette demonstrate that adoptive transfer of allergy-resistant rats AMs to allergy-sensitive rats mitigated airway hyperresponsiveness[533]. However further study is required before it may be considered in the context of lung injury.

Mitochondrial transfer from MSCs through the use of TNTs, gap junctions or EVs to a number of cell types including macrophages has been reported[115, 116, 291, 318]. This exchange has been associated with protective effects in lung injury through the enhancement in alveolar epithelial cell bioenergetics[318]. Our group previously reported macrophages receiving MSC mitochondria via TNTs demonstrate enhanced phagocytosis[116]. Mitochondria are responsible for oxidative metabolism which is known to play a role in modulating macrophage function. Vats *et al* demonstrate that IL-4 induces fatty acid oxidation in macrophages which was required for enhancement of arginase activity (M2 marker) and suppression of LPS-induced TNFα production[240]. In the current study, EV-mediated mitochondrial transfer from hMSCs to hMDMs was demonstrated by flow cytometry and fluorescence imaging. Preliminary data suggests an increase in hMDM mitochondrial respiration with hMSC-CM which was prevented with the use of hMSCs with dysfunctional mitochondria. Inhibiting oxidative phosphorylation in hMDMs or generating dysfunctional hMSC mitochondria both prevented the enhancement of phagocytosis and the suppression of TNFα secretion normally seen with hMSC-CM treatment. Taken together these results indicate that hMSC mitochondrial transfer to hMDMs is required to produce these effects. These findings are also in keeping with the study produced by Vats *et al* demonstrating the link between oxidative respiration and macrophage alternative activation. The next step for this line of research would be to confirm that it is mitochondrial transfer to AMs that is responsible for the protective effects of hMSC-EVs in lung injury *in vivo*.

Collectively, the results obtained from this study show for the first time that hMSCs produce an M2-like human macrophage phenotype in the presence of ARDS patient BALF which is defined by decreased inflammatory cytokine secretion and enhanced phagocytic capacity. Mechanistic experiments indicate that hMSC-EV-mediated transfer of mitochondria is responsible for the modulation of human macrophage phenotype through promotion of mitochondrial oxidative phosphorylation.

## 6.2 Future directions

The current work has provided further insight into the complex mechanisms of effect of hMSCs in lung injury, specifically through their interactions with macrophages. Further study into understanding the mechanism of hMSC modulation of human macrophages could focus on uncovering the pathways involved in induction of the M2-like macrophage phenotype in response to mitochondrial transfer. As discussed earlier, the literature has already made clear the association between metabolism and macrophage polarisation[242, 534]. Vats *et al* show the importance of oxidative phosphorylation in the acquisition of an IL-4-induced M2 macrophage phenotype[240]. They demonstrate that IL-4 signals through STAT6 and PGC-1β to promote fatty acid oxidation which was important for modulating macrophage function. The current study supports this finding and suggests that mitochondrial transfer from hMSCs to the hMDMs promotes this phenotypic shift through promotion of oxidative phosphorylation. It is possible that the donation of hMSC mitochondria to the hMDMs could represent a more direct way of modulating metabolic activity and macrophage function. It would be worthwhile investigating the effects of hMSC-EVs on STAT6 signalling and activation of PGC-1β to determine if the signalling pathways involved in the hMSC-induced phenotype and the IL-4 induced phenotype are similar. It would be interesting to investigate these mechanisms further *in vivo*. The results demonstrate that treatment of lung injury with hMSC-EV-treated AMs is protective but they do not show that it is mitochondrial transfer through these EVs that is responsible. Treatment of AMs with rhodamine 6G pre-treated hMSC-EVs would provide evidence that it is the mitochondria-containing EVs that are conferring these protective effects to the AMs.

Another question that remains is exactly how hMSC-EV treated AMs are protective in this lung injury model. Characterisation of the secretory profiles of the hMDMs *in vitro* showed a general downregulation of both pro and anti-inflammatory mediators. Presumably, the induction of an M2-like phenotype in these AMs would potentially dampen inflammation by influencing host AMs to reduce their inflammatory cytokine secretion or even adopt a similar M2-like profile. The cytokines produced by the EV-treated AMs were not investigated and the *in vitro* experiments in hMDMs would

suggest that neither IL-10 nor IL-1ra are responsible for the anti-inflammatory effects of these AMs. Investigation of the secretory profile of hMSC-EV-treated macrophages may uncover the mediator responsible and uncover a new therapeutic candidate for the treatment of lung injury. The findings in the present study add to the accumulating evidence that hMSC-EVs can mediate many of the therapeutic effects of the whole cell therapy itself. Recently there is an increasing interest in the use of MSC-EVs as a treatment itself for a number of conditions including lung injury, pulmonary hypertension, kidney injury and stroke[113, 171, 535, 536]. Certainly, EV preparations have been investigated in clinical trial and appears to be a feasible prospect[322]. hMSC-EVs may be able to overcome a number of the hurdles associated with hMSC therapy; EVs avoid the risk of transformation that is associated with stem cell therapies; EVs have very low expression of MHC class I and II on their surfaces meaning minimal immunogenicity, an emerging concern with hMSCs as a cell therapy[48]. One potential disadvantage however is that EVs will not be able to actively respond to their environment in the way that MSCs do, although there is evidence that they may not need to. Certainly, studies reporting the efficacy of hMSC-EVs often use preparations which are constitutively produced by MSCs with no pre-conditioning of any kind[112, 171, 537]. This is not to suggest that the cells could not be stimulated *in vitro* to modulate their EV production in order to optimise their therapeutic effect *in vivo*. Serum starvation is reported to enhance MSC-EV production[538]. Bustos *et al* showed that hMSCs which were pre-conditioned with ARDS patient serum were more protective than unconditioned hMSCs in endotoxin-induced lung injury[137]. It is possible that such pre-conditioning regimens could influence EV production and contents enhancing their effects. hMSC-EVs are a promising candidate to replace hMSCs as a therapy for ARDS but our understanding of this cell product and their mechanisms is limited.

Reliable potency assays which may be used to inform the release of MSCs for use in clinical trials remain elusive. Bloom *et al* tested the robustness and feasibility of testing MSC suppression of mixed lymphocyte reactions as a potency assay[539]. The observation that AMs are cellular mediators of their therapeutic effects proposes that the capacity of hMSCs to modulate macrophage phenotype could be assessed and serve as a potency assay. In this study CD206 was upregulated by hMDMs in ARDS

BALF which was associated with reduced TNFα secretion and enhanced phagocytosis. It may be worth testing whether MSC enhancement of macrophage expression of CD206 correlates with their ability to dampen macrophage inflammatory cytokine secretion or enhance phagocytic activity. If so, assessment of CD206 expression in hMSC-cultured monocytes or macrophages could serve as a potency assay for the selection of MSC donors for use in clinical trials in ARDS as well as a biomarker to assess their efficacy after treatment.

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